

MECHANISMS OF HUMAN NEUTROPHIL PRIMING

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Work presented in this thesis was carried out solely by the author, unless otherwise stated, from 1st November 1992 to 1st November 1995 under the supervision of Professor Chris Haslett and Dr. Edwin Chilvers, Rayne Laboratory, University of Edinburgh.

A handwritten signature in black ink, appearing to read 'A.M. Condcliffe'. The signature is fluid and cursive, with the first letters of the first and last names being capitalized and prominent.

Alison M. Condcliffe.

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Abstract

Neutrophils have been implicated in the pathogenesis of many human diseases, including the adult respiratory distress syndrome (ARDS), pulmonary fibrosis and ischaemia-reperfusion injury. Priming of neutrophils by pre-exposure to bacterial products and inflammatory mediators represents a potent means of augmenting both the bactericidal capacity and injurious potential of these cells; in particular, the generation of reactive oxygen species such as the superoxide anion (O_2^-) may be enhanced by up to twenty-fold. Greater understanding of the signalling mechanisms underlying neutrophil priming could lead to novel therapeutic strategies aimed at preventing neutrophil-mediated tissue injury in these conditions.

Exposure of neutrophils to the priming agents lipopolysaccharide (LPS), tumour necrosis factor- α (TNF α) and platelet-activating factor (PAF) was shown to modulate adhesion molecule expression and function (in particular inducing upregulation of β_2 integrins and shedding of L-selectin) in an agonist-specific fashion, with a time-course which correlated with that required to establish the primed state.

Neutrophil chemoattractant receptors are coupled to intracellular signalling pathways by heterotrimeric G-proteins, principally $G_{i\alpha 2}$. I have shown that TNF α , LPS and PAF increase the level of $G_{i\alpha 2}$ expression detectable in neutrophil membranes, the time-course for this effect mirroring that for priming. However, the degree of G-protein upregulation is considerably less than the enhancement of the respiratory burst, suggesting that downstream signalling events play a more critical mechanistic role in priming. Two major signalling pathways implicated in the activation of the neutrophil respiratory burst were studied; the cleavage of the membrane phospholipid phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P₂) by phospholipase C to

yield inositol 1,4,5-trisphosphate (Ins(1,4,5)P₃), and its phosphorylation by phosphatidylinositol 3-hydroxykinase (PI3K) to phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P₃). Accumulation of neutrophil Ins(1,4,5)P₃ (the signal for intracellular calcium release) in response to the secretagogue N-formyl-methionyl-leucyl-phenylalanine (fMLP) was identical in unprimed and primed neutrophils, suggesting that modulation of phospholipase C activity is not involved in signalling neutrophil priming. A small but significant enhancement of fMLP-stimulated PtdIns(3,4,5)P₃ accumulation (about 25%) was seen at 10 s in TNF α -primed versus unprimed cells, but 60 s following fMLP stimulation this enhancement was 620%. This suggests that enhanced and sustained PtdIns(3,4,5)P₃ generation may be important in signalling mechanisms leading to the primed respiratory burst.

Abbreviations

ACLB	Albumin-coated latex beads
ARDS	Adult respiratory distress syndrome
ATP	Adenosine triphosphate
BAC	Bovine adrenal cortex
BAL	Bronchoalveolar lavage
BCA	Binchinoic acid
BSA	Bovine serum albumin
Ca ²⁺	Calcium ion
[Ca ²⁺] _i	Intracellular free calcium concentration
cADPR	Cyclic adenosine diphosphate-ribose
CGD	Chronic granulomatous disease
C5a	Complement fragment 5a (anaphylatoxin)
DAG	Diacylglycerol
DMSO	Dimethyl sulfoxide
EDTA	Ethylenediamine tetraacetic acid
EGTA	Ethylene glycol (B-aminoethylether)-N,N'-tetraacetic acid
FAK	Focal adhesion kinase
FcγRIII	Immunoglobulin Fc receptor 3
FITC	Fluorescein isothiocyanate
fMLP	N-formyl-methionyl-leucyl-phenylalanine
G-protein	Guanine nucleotide binding protein
GDI	Guanine nucleotide dissociation inhibitor
GDP	Guanine diphosphate
GM-CSF	Granulocyte-macrophage colony-stimulating factor

GTP	Guanine triphosphate
HEPES	<i>N</i> -2-hydroxyethylpiperazine- <i>N</i> -ethane sulphonic acid
HL-60	Human leukemia cell line
ICAM-1	Intercellular cell adhesion molecule-1
IFN γ	Interferon- γ
Ins(1,4,5)P ₃	Inositol 1,4,5-trisphosphate
InsP ₆	Inositol hexakisphosphate
IL-1	Interleukin-1
IL-6	Interleukin-6
IL-8	Interleukin-8
LAD	Leukocyte adhesion deficiency
LBP	Lipopolysaccharide-binding protein
LPS	Lipopolysaccharide
LTB ₄	Leukotriene B ₄
MAPK	Mitogen activated protein kinase
MAPKK	MAPK kinase
MARCKS	Myristoylated alanine rich C kinase substrate
mAb	Monoclonal antibody
NADP	Nicotinamide adenine dinucleotide phosphate
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
NF-kB	Nuclear factor-kB
O ₂ ⁻	Superoxide anion
PA	Phosphatidic acid
PAF	Platelet-activating factor
PAGE	Polyacrylamide gel electrophoresis

PBS	Phosphate-buffered saline
PC	Phosphatidylcholine
Phox	Phagocyte oxidase
Pi	Inorganic phosphate
PI	Phosphatidylinositol
PI3K	Phosphatidylinositol 3-hydroxykinase
PIC	Phosphoinositide-specific phospholipase C
PLA ₂	Phospholipase A ₂
PLD	Phospholipase D
PKC	Protein kinase C
PMA	Phorbol myristate acetate
PPP	Platelet-poor plasma
PRP	Platelet-rich plasma
PtdIns3P	Phosphatidylinositol 3-phosphate
PtdIns4P	Phosphatidylinositol 4-phosphate
PtdIns(3,4)P ₂	Phosphatidylinositol 3,4-bisphosphate
PtdIns(4,5)P ₂	Phosphatidylinositol 4,5-bisphosphate
PtdIns(3,4,5)P ₃	Phosphatidylinositol 3,4,5-trisphosphate
ROI	Reactive oxygen intermediate
SAPK	Stress-activated protein kinase
SDS	Sodium dodecyl sulphate
SOD	Superoxide dismutase
TNF α	Tumour necrosis factor- α
TRAF	TNF receptor associated factor

CONTENTS.

PBS	Phosphate-buffered saline
PC	Phosphatidylcholine
Phox	Phagocyte oxidase
Pi	Inorganic phosphate
PI	Phosphatidylinositol
PI3K	Phosphatidylinositol 3-hydroxykinase
PIC	Phosphoinositide-specific phospholipase C
PLA ₂	Phospholipase A ₂
PLD	Phospholipase D
PKC	Protein kinase C
PMA	Phorbol myristate acetate
PPP	Platelet-poor plasma
PRP	Platelet-rich plasma
PtdIns3P	Phosphatidylinositol 3-phosphate
PtdIns4P	Phosphatidylinositol 4-phosphate
PtdIns(3,4)P ₂	Phosphatidylinositol 3,4-bisphosphate
PtdIns(4,5)P ₂	Phosphatidylinositol 4,5-bisphosphate
PtdIns(3,4,5)P ₃	Phosphatidylinositol 3,4,5-trisphosphate
ROI	Reactive oxygen intermediate
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Chapter 1: Introduction

1.1 The Neutrophil - General

Following its discovery by Ehrlich in 1879 the neutrophil has been recognised as the most numerous of the circulating phagocytes, constituting in most species approximately half the circulating white cell population; its fundamental importance in host defence is emphasised by the fact that, phylogenetically, it emerged long before the lymphocyte. Studies using radiolabelled neutrophils suggest that they have a short circulating half-life (about 6.6 hrs) in the blood (Mauer et al., 1960). Neutrophils are recruited rapidly and in large numbers to sites of infection or inflammation, where they respond to the injurious agent(s) by phagocytosis of microorganisms, by the release of preformed toxic enzymes and proteins from an array of granules, and by the de novo production of a range of potentially damaging but ephemeral reactive oxygen intermediates (ROI). That these functions are essential to host defence is illustrated by the marked propensity to develop infection seen in certain deficiency syndromes, discussed below (section 1.5). Paradoxically, it is believed that the inappropriate or excessive release of these compounds may also contribute to inflammatory tissue injury in a variety of disease states (section 1.6).

The neutrophil has previously been regarded as an unsubtle, immutable vehicle, existing in either a fully quiescent or a fully activated state, recruited in bulk to inflammatory foci to deliver its toxic products and to die. More recent research has revealed that the neutrophil, although an end-stage cell, is complex and variable in its functional responses. In particular, neutrophil functions can be regulated by environmental factors, and their secretory/injurious potential greatly amplified by a variety of priming agents; these include bacterial products (such as lipopolysaccharide (LPS) and the

formylated peptides), cytokines (such as tumour necrosis factor- α (TNF α), interleukin-8 (IL-8), granulocyte macrophage-colony stimulating factor (GM-CSF) and interferon- γ (IFN γ)), and lipid mediators (e.g. platelet activating factor (PAF) and leukotriene B₄ (LTB₄)). These mediators, generated in the vicinity of inflammatory sites, both stimulate an influx of neutrophils by chemotaxis and modulate their effector functions, ideally eliminating the injurious stimulus without damage to host tissue. However, as neutrophil priming may also increase neutrophil-mediated tissue injury, the dissection of priming mechanisms may lead to a fuller understanding of the pathogenesis of host tissue damage, and ultimately to the development of novel therapeutic strategies to reduce such injury.

Very low concentrations (10^{-10} - 10^{-9} M) of chemotactic agents such as the complement component C5a, the formylated peptide N-formyl-methionyl-leucyl-phenylalanine (fMLP) and the chemokine IL-8, induce neutrophil shape-change with polarisation and chemotaxis. Directed migration leads to the exposure of the neutrophil to progressively higher concentrations of inflammatory mediators, resulting in priming and ultimately full activation. Activated chemotactic receptors couple to GTP-binding proteins (G-proteins), stimulating a variety of cellular signal transduction processes (see Figure 1.1). Activation of phosphatidylinositol-specific phospholipase C (PIC) results in the cleavage of the membrane phospholipid phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P₂) to yield diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (Ins(1,4,5)P₃). Ins(1,4,5)P₃ diffuses into the cytosol and induces the release of Ca²⁺ from intracellular stores (Streb et al., 1983) with consequent influx of Ca²⁺ through the plasma membrane, and a transient increase in cytosolic calcium concentration ([Ca²⁺]_i). The lipid DAG remains membrane-associated; DAG is also generated from phosphatidylcholine (via phosphatidic acid, PA) by

Figure 1.1

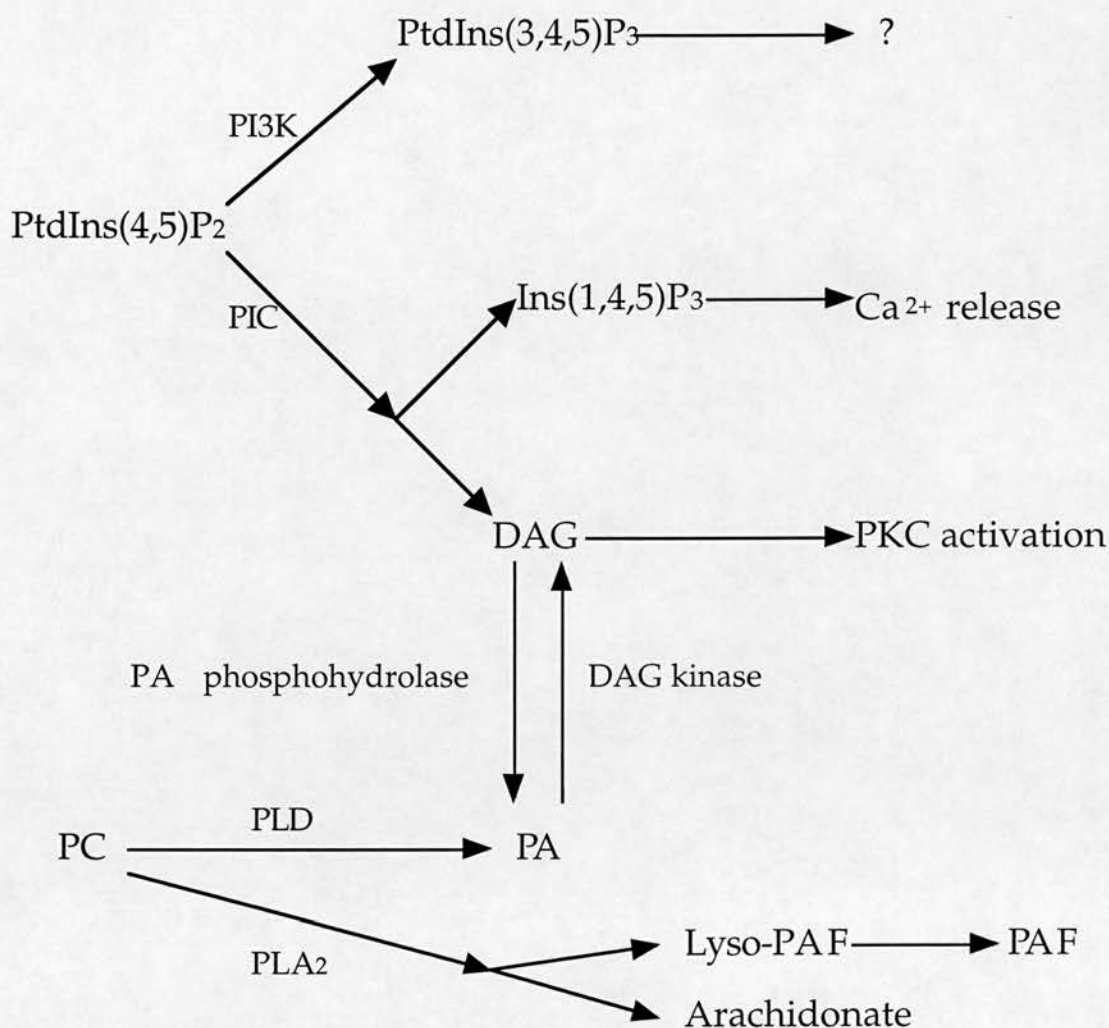


Figure 1.1 Signalling Pathways in Activated Neutrophils

PtdIns(4,5)P₂ acts as a substrate for both PI3K and for PIC, yielding PtdIns(3,4,5)P₃ (physiological role(s) uncertain) and both Ins(1,4,5)P₃ (the messenger for intracellular Ca²⁺ release) and the PKC activator DAG (also derived from PA, cleaved from phosphatidylcholine (PC) by PLD), respectively. PAF and arachidonate are derived from PC by the action of phospholipase A₂.

phospholipase D, and activates members of the protein kinase C family (Nishizuka, 1984) which phosphorylate components of the oxygen radical-generating system. G-protein coupled receptors also activate phospholipase A₂ (PLA₂), leading to the generation of arachidonate, lyso-PAF and hence PAF (Chilton et al., 1984). Arachidonate can activate the NADPH oxidase in cell-free systems. The activation of various enzymes is regulated by phosphorylation of critical tyrosine residues (see section 1.8.7). Finally, the recently described phosphatidylinositol 3-hydroxykinase (PI3K) can phosphorylate PtdIns(4,5)P₂ to generate the putative second messenger phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P₃). Priming agents could therefore be acting at the level of the receptors, G-proteins, signalling pathways, tyrosine phosphorylation or effector mechanisms (NADPH oxidase) to produce the enhanced responsiveness characteristic of the inflammatory neutrophil.

1.2 Neutrophil Granules

Much of the destructive potential of the neutrophil relates to the proteases and microbicidal factors localised within a variety of granules; additionally, several adhesion molecules and chemotactic factor receptors stored in the granule membranes can be mobilised to the plasma membrane upon exocytosis. Differences in granule composition distinguish neutrophils from other granulocytes. The traditional classification of neutrophil granules as peroxidase-positive (azurophil, or primary) or peroxidase-negative (specific or secondary) has proved too simple to account for the observed differential exocytosis of granule protein and incorporation of granule membrane into plasma membrane, and a new classification has been formulated (Borregaard et al., 1993).

1.2.1 Secretory Vesicles

Secretory vesicle membranes contain the adhesion receptor CD11b/CD18 (Mac-1), essential for integrin-mediated neutrophil adhesion (Sengelov et al., 1993a, Calafat et al., 1993) in addition to the fMLP-receptor (Jesaitis et al., 1982), alkaline phosphatase, and cytochrome b₅₅₈ (the membrane component of the NADPH oxidase, Calafat et al., 1993); decay accelerating factor may also be present (Berger et al., 1987). Secretory vesicles are the most readily and rapidly mobilised of the neutrophil granules (Sengelov et al., 1993b), providing prompt upregulation of cell surface CD11b/CD18 (promoting adhesive events) and fMLP receptors (supplying the capacity to augment cellular chemoattractant responsiveness).

1.2.2 Specific (Secondary) Granules

These spherical or rod-shaped granules are defined by their content of lactoferrin; they are also an important store of cytochrome b₅₅₈ (Borregaard et al., 1983, Borregaard and Tauber, 1984), FcRIII (receptors for the Fc portion of antibodies, Tosi et al., 1992), adhesion molecules (Bainton et al., 1987) and fMLP receptors (Fletcher and Gallin, 1983).

1.2.3 Gelatinase (Tertiary) Granules

These granules are smaller than the specific granules and constitute approximately 25% of all peroxidase-negative granules. They are principally composed of the protease gelatinase, which may be involved in digesting the vascular basement membrane to allow neutrophil extravasation (Weiss and Peppin, 1986)

1.2.4 Azurophilic (Peroxidase-Positive) Granules

Azurophil granules store the majority of the proteolytic and bactericidal proteins (myeloperoxidase, cathepsins, elastase, β -glucuronidase, lysozyme, defensins etc.) with no receptors or adhesion molecules demonstrated in their membranes. Azurophil granules are mobilised slowly, and generally only in a limited fashion.

1.3 The NADPH Oxidase and the Respiratory Burst

During phagocytosis, neutrophils (and other phagocytes) exhibit an intense burst of oxygen consumption (Balldridge and Gerard, 1933) known as the respiratory burst, which is essential for the killing of ingested microorganisms. The net reaction of the burst is the transfer of electrons from NADPH (generated by the hexose monophosphate shunt) to oxygen, producing superoxide anions (O_2^-). The enzyme system which catalyses this reaction is known collectively as the NADPH oxidase; defects within this system lead to the condition of chronic granulomatous disease (CGD), characterised by increased susceptibility to bacterial infection. Study of the neutrophils of patients suffering from this condition has yielded much information concerning the individual components of the NADPH oxidase and their functions (reviewed by Thrasher et al., 1994).

1.3.1 Cytochrome b_{558}

Cytochrome b_{558} is located within the plasma membrane and in the membranes of peroxidase-negative granules. It consists of an α - and a β -subunit, designated $p21^{phox}$ and $p91^{phox}$ respectively according to their molecular weights (phox - *phagocyte oxidase*). Absence of the cytochrome is the commonest molecular lesion underlying CGD (Casimir et al., 1992); both subunits are required for mutual stability. In the majority of cases, CGD is

X-linked, the gene coding for p91^{phox} being located on the X chromosome. A small number of patients lacking cytochrome b₅₅₈ do so as a consequence of an autosomal recessive inheritance of a defective p21^{phox}. The binding site for NADPH has been shown to reside in the p91^{phox} component of the oxidase (Rotrosen et al., 1992); indeed, the entire electron transport chain from NADPH to oxygen is contained within the cytochrome b₅₅₈, although cytoplasmic factors are essential for its activation/regulation (section 1.3.2).

1.3.2. Cytosolic components of the NADPH Oxidase: p47^{phox}, p67^{phox} and p40^{phox}

One third of patients with CGD display an autosomal recessive pattern of inheritance, the majority having a normal flavocytochrome and lacking one of two cytosolic proteins. p47^{phox} was discovered as a protein which became phosphorylated on activation of normal phagocytes but was absent from the neutrophils of most of these patients (Segal et al., 1985); p67^{phox} was subsequently shown to be missing from the small number of remaining patients (Volpp et al., 1988). The small GTP-binding protein p21^{rac} is also thought to be vital for oxidase activation, since it is essential to reconstitute oxidase activity in cell-free assays (see 1.3.3). Oxidase activity can be reconstituted with recombinant p47^{phox}, p67^{phox}, p21^{rac} and purified (Abo et al., 1992) or recombinant (Rotrosen et al., 1993) cytochrome b₅₅₈.

p47^{phox} is phosphorylated in discrete steps on at least seven serine residues (El Benna et al., 1994), and the phosphorylation events have been correlated with activation of the oxidase (Okamura et al., 1988, Rotrosen and Leto, 1990). More recently, p67^{phox} was also shown to become phosphorylated (Dusi and Rossi, 1993). Both factors translocate to the plasma membrane when cells are activated (Clark et al., 1990). Two lines of evidence suggest that their docking site is the flavocytochrome itself; firstly, translocation does

Figure 1.2

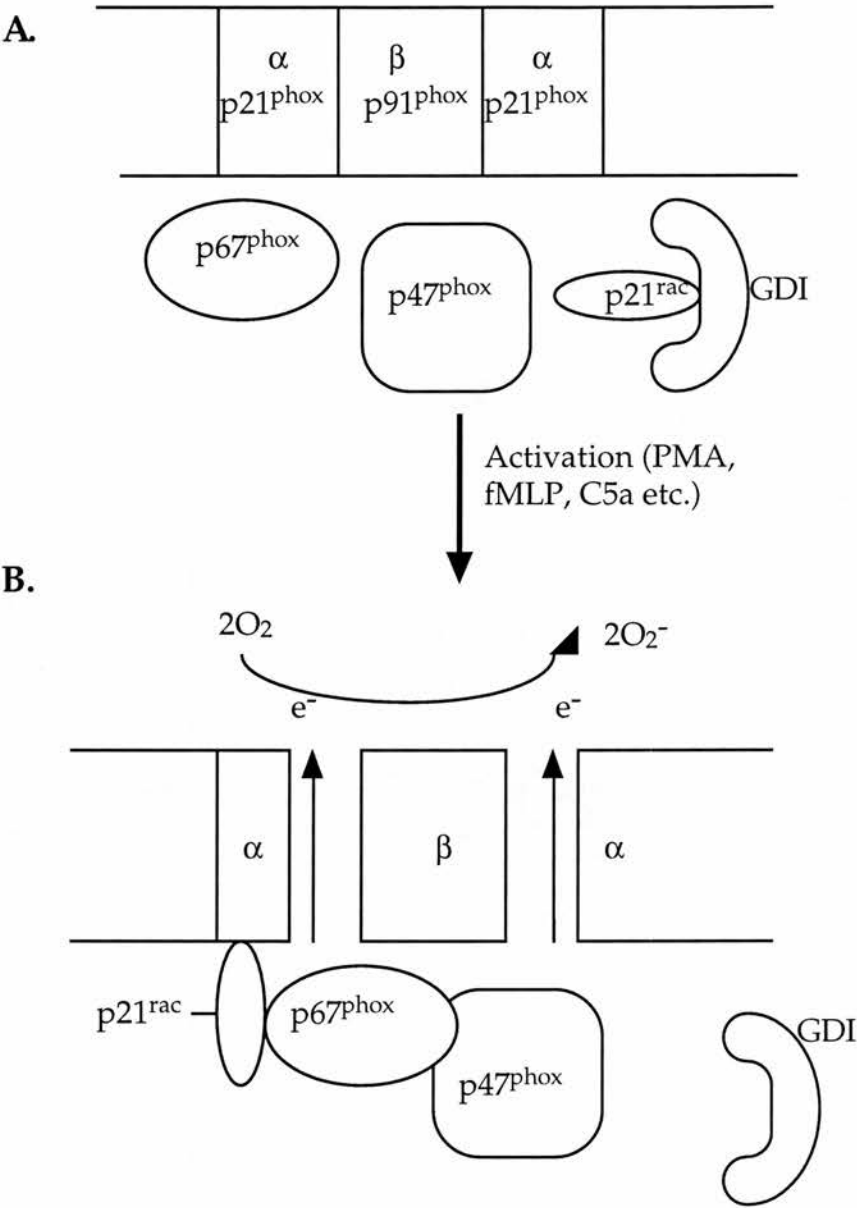


Figure 1.2 NADPH Oxidase Assembly

Agonist stimulation results in assembly of the NADPH oxidase from (A) its component parts (the flavocytochrome b_{558} subunits p91^{phox} and p21^{phox} and the cytosolic factors p47^{phox}, p67^{phox} and p21^{rac}) to the fully functional enzyme (B).

not occur when the cytochrome is absent in X-linked CGD (Heyworth et al., 1991), and secondly, peptides corresponding to the C-termini of the flavocytochrome inhibit this translocation (Rotrosen et al., 1990, Nakanishi et al., 1992).

A further protein which has strong homology with p47^{phox}, p40^{phox}, has been found to co-immunoprecipitate with p67^{phox} (Wientjes et al., 1993). p40^{phox} is not required for activity in the cell-free system, and as yet no CGD patients have been identified who lack this protein, hence its function is speculative.

1.3.3 Cytosolic Components of the NADPH Oxidase: Small G-proteins

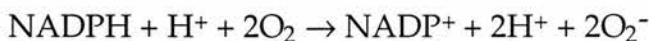
The ras-related family of small G-proteins act as molecular switches, with the bound nucleotide modulated between the GTP -on and GDP -off modes by other regulatory molecules. One such protein, p21^{rac}, is required for reconstitution of respiratory burst activity in the cell-free system. Upon stimulation of neutrophils, p21^{rac} dissociates from its complex with a GDI (GDP-dissociation inhibitor) and translocates to the membrane simultaneously with p47^{phox} and p67^{phox} (Quinn et al., 1993, Abo et al., 1994). Reduction of p21^{rac} activity by anti-sense oligonucleotides in B lymphocytes was shown to diminish oxidase activity (Dorsueil et al., 1992). Additionally, increased oxidase activity was reported in bcr-knockout mice (Voncken et al., 1995); bcr is a GTPase-activating protein for p21^{rac}, and diminution of bcr would be expected to result in higher levels of active p21^{rac}.

The precise role of p21^{rac} in oxidase activation remains to be elucidated. Its role in actin microfilament reorganisation during membrane ruffling (Ridley et al., 1992) suggests an involvement with the cytoskeleton, while the recently described association of p21^{rac} with p21-activated kinase (Prigmore

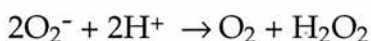
et al., 1995) indicates a possible action via a downstream kinase. Neither of these possibilities, however, explain the need for p21^{rac} in cell-free systems.

1.3.4 Production of Reactive Oxygen Intermediates

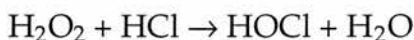
The NADPH oxidase catalyses the following reaction:



NADPH produced by the cytosolic hexose monophosphate shunt functions as an electron donor to effect a one electron reduction of each of two atoms of molecular oxygen (Babior et al., 1973). The O_2^- so formed dismutates to form hydrogen peroxide, a reaction catalysed by superoxide dismutase:



In the presence of halide ions (preferentially chloride ions) and H_2O_2 , myeloperoxidase released from neutrophil azurophilic granules can catalyse the generation of hypohalous acids, such as hypochlorous acid:



Cytotoxic effects attributable to hypochlorous acid include: oxidation/decarboxylation of membrane proteins, leading to increased bacterial cell permeability (Albrich et al., 1986), oxidation of components of the bacterial respiratory chain (Rakita et al., 1989), membrane peroxidation (Winterbourn et al., 1992) and formation of chloramine (Bernofsky, 1991). Even more reactive products such as hydroxyl radical and singlet oxygen may also be formed, but they are so short-lived that detection is difficult and their role uncertain (reviewed in Rosen et al., 1995)

1.4 Other Neutrophil Functions

1.4.1 Phagocytosis

Once a neutrophil has migrated into the tissues, its primary purpose is to recognise, phagocytose and destroy microorganisms. Phagocytosis consists of two steps - recognition, and ingestion into the phagosome. In most cases, recognition is dependent on opsonisation by specific (immunoglobulins) or non-specific (complement components) plasma proteins which bind neutrophil cell surface receptors. For engulfment of an opsonised particle to occur, pseudopods rich in filamentous actin must form and surround the particle; this requires actin polymerisation and is inhibited by agents such as cytochalasins which block this latter process (Zigmond and Hirsch, 1972). During phagocytosis, the oxidative burst is initiated (1.3) and cytosolic granules (1.2) fuse with the invaginating plasma membrane, creating a highly toxic microenvironment. Other metabolic pathways are also activated by phagocytosis (Della Bianca et al., 1993) including the synthesis of bioactive lipids (see 1.4.2) and of cytokines (1.4.3)

1.4.2 Synthesis of Bioactive Lipids

Activation of 1-*O*-alkyl PC-specific PLA₂ liberates lyso PAF and arachidonic acid from the membrane phospholipid 1-*O*-alkyl-2-arachidonyl-*sn*-glycero-3-phosphocholine (Chilton et al., 1984); transfer of acetate from acetyl CoA to lyso-PAF yields PAF, whilst arachidonic acid may be metabolised to LTA₄ and LTB₄. Neutrophils synthesise LTB₄ in response to a variety of stimuli, including fMLP (Clancy et al., 1983); LTB₄ stimulates many neutrophil functions, including chemotaxis (Palmlblad et al., 1981), calcium mobilisation (Nacchache and Sha'afi, 1983) and degranulation (Bokoch and Reed, 1981).

The effects of PAF on neutrophil functions are considered in greater detail below (1.7.4).

1.4.3 Cytokine Production

Protein synthesis by neutrophils can be elicited by a variety of agonists including fMLP, TNF α and GM-CSF (Beaulieu et al., 1992), and several cytokines are produced including interleukin-1 (IL-1) (Tiku et al., 1986), IL-8 (Bazzoni et al., 1991) and TNF α (Dubravek et al., 1990). Because of the large numbers of neutrophils recruited to inflammatory foci, the impact of cytokine synthesis by these cells may be highly significant.

1.5 The Role of the Neutrophil in Host Defence: Neutrophil Dysfunction

Neutrophil disorders may be asymptomatic or characterised by recurrent infections, especially with pyogenic bacteria. Abnormalities may be quantitative (neutropenia) or qualitative (disorders of adhesion, chemotaxis, phagocytosis or killing), and they may be congenital or acquired. Gram-negative bacilli such as *Pseudomonas aeruginosa*, *Klebsiella* species and *Escherichia coli*, Gram-positive cocci (Streptococci and Staphylococci) and fungi (*Candida* species and *Pneumocystis carinii*) are common causes of infection in patients with neutrophil dysfunction.

1.5.1 Neutropenia

The hallmark study of Bodey et al. (1966) demonstrated that the risk of infection in granulocytopenic patients increases rapidly when the granulocyte count falls to below $0.5 \times 10^9/l$, with the majority of life-threatening infections occurring at $<0.1 \times 10^9/l$. Neutropenia may occur as an isolated deficiency or be associated with other cytopenias, as in aplastic anaemia. Neutropenia may be secondary to treatment with cytotoxic drugs,

autoimmune destruction, or may be congenital, constitutional or idiopathic. Drug-induced neutropenia is by far the commonest group seen in medical practice.

1.5.2 Disorders of Leukocyte Migration

1.5.2.1 Defects of Chemotaxis

Neutrophils respond to low concentrations of chemotactic agents such as C5a and fMLP by undergoing polarisation and directed locomotion along the chemotactic gradient, facilitating recruitment to inflammatory sites. In a study of 94 patients with recurrent infections localised to the respiratory tract or skin, Ottonello et al. (1995) found disordered chemotaxis in 41 cases, suggesting that this may be a relatively common abnormality. Defects were found in response to either agent individually or to both; the molecular mechanisms underlying the disorders were not investigated.

1.5.2.2 Leukocyte Adhesion Deficiency

Just as the study of patients with chronic granulomatous disease has helped to elucidate the mechanisms underlying the respiratory burst, so the molecular mechanisms of neutrophil adhesion to the endothelium have been clarified by the study of various leukocyte adhesion deficiency (LAD) states. The patients suffer recurrent life-threatening bacterial and fungal infections, with a persistent peripheral blood granulocytosis and a failure of neutrophils to migrate to sites of infection. In the majority of cases (those with LAD type I, Anderson and Springer, 1987) the underlying anomaly is a mutation in the gene coding for the common β subunit (CD18) of the neutrophil β_2 integrins, which mediate firm adhesion to the endothelial cells (1.8.6) by binding to their counter-receptor, intercellular adhesion molecule-1 (ICAM-1). A minority of patients have been described with an identical clinical syndrome

but normal integrin structure and function; in these cases, designated LAD type II, the abnormality lies in the glycosylation of a family of glycoproteins, the selectins, which mediate the initial transient contacts of the neutrophil with the vascular endothelium (Phillips et al., 1995). Neutrophil adhesion is discussed in greater detail in Chapter 4.

1.5.3 Disorders of Killing

1.5.3.1 Granule Abnormalities

The commonest disorder affecting neutrophil granules is hereditary myeloperoxidase deficiency, which occurs in 1/2000-1/5000 people and is not usually associated with any clinical abnormality or with increased susceptibility to infection (Kitahara et al., 1981). In contrast, the rare Chediak-Higashi syndrome, an autosomal recessive condition characterised by abnormally large neutrophil granules and albinism, is associated with a predisposition to infection (Rausch et al., 1978).

1.5.3.2 Chronic Granulomatous Disease

As discussed above (1.3) this condition results from abnormalities in the various components of the NADPH oxidase, with resultant failure to generate a respiratory burst on phagocytosis (reviewed in Thrasher et al., 1994). Neutrophils migrate normally and phagocytose microorganisms, but there is a failure of intracellular killing. Clinically, the syndrome is characterised by recurrent bacterial and fungal infections, particularly at epithelial surfaces in direct contact with the environment (skin, mucous membranes, lung and gut); histology reveals widespread granulomatous infiltration of tissues, probably resulting from the inability to eliminate infectious agents. Diagnosis is made by the failure of neutrophils to oxidise

nitroblue tetrazolium on stimulation with the phorbol ester, PMA (phorbol myristate acetate).

This disease results in serious morbidity and early death in many affected individuals. Treatment is with antibiotics (both prophylactically and to treat intercurrent infection), antifungals, the administration of immunodulatory cytokines (particularly interferon- γ), and in some cases bone marrow transplantation.

1.6. The Role of Neutrophils in Tissue Destruction

The neutrophil has been increasingly implicated as a mediator of tissue injury in a variety of inflammatory conditions such as the adult respiratory distress syndrome (ARDS) (reviewed by Donnelly and Haslett, 1992), pulmonary fibrosis (e.g. Behr et al., 1991), vasculitic diseases (reviewed by Savage and Rees, 1994) and ischaemia-reperfusion injury (reviewed by Williams, 1994). Although the neutrophil's arsenal of toxins normally defends the host against invading microorganisms, it has no intrinsic ability to differentiate between foreign and host antigens and must rely on the specific arms of the immune response (antibodies, and other cells) to select its targets. If normal host tissues are wrongly identified as foreign, or if inflammatory stimuli overwhelm the normal controls preventing generalised activation, the neutrophil's destructive potential will be inappropriately elicited. The role of the neutrophil has been studied in great depth in the adult respiratory distress syndrome (ARDS), which serves as a good model for other disease states in which the neutrophil is a key player.

1.6.1 The Neutrophil in ARDS

ARDS is a form of acute lung injury characterised by high permeability pulmonary oedema and refractory hypoxaemia (Ashbaugh et al., 1967)

following a wide variety of pulmonary and non-pulmonary insults (e.g. pneumonia, septicaemia, pancreatitis, multiple trauma and gastric aspiration; Fowler et al., 1983). Despite improvements in supportive care, no effective specific treatment exists and the mortality from the condition remains at 50-70% (Rocker et al., 1989); hence the underlying inflammatory mechanisms are being studied to identify potential therapeutic avenues.

Both morphologic (Bachofen and Weibel, 1977) and bronchoalveolar lavage (BAL) studies (Fowler et al., 1987, Lee et al., 1981) have demonstrated that neutrophils are present in large numbers in the lower respiratory tract of patients with ARDS; Weiland et al. (1986) have correlated the percentage of neutrophils obtained at lavage with the severity of lung injury. Additionally, Zimmerman et al. (1983) found evidence of activated neutrophils in the pulmonary circulation of patients with ARDS. The neutrophil proteases elastase (Lee et al., 1981) and collagenase (Christner et al., 1985) have been identified in excess in BAL fluid; these enzymes, and hydrogen peroxide found in expired air from ARDS patients receiving assisted ventilation (Baldwin et al., 1986) are thought to derive from activated inflammatory cells within the damaged lung. Neutrophil proteases and ROI may interact to cause tissue injury; for example, Cochrane et al. (1983) demonstrated oxidant-induced inactivation of α -1 proteinase inhibitor, which neutralises neutrophil elastase, in the BAL fluid from patients suffering from ARDS.

Multiple animal models of ARDS also support a pathogenic role for the neutrophil. Drug-induced neutropenia decreased the severity of lung injury induced by circulating endotoxin (Heflin and Brigham, 1981), hyperoxia (Shasby et al., 1982a) and microembolisation (Flick et al., 1981, Johnson and Malik, 1980). Isolated lungs became oedematous when perfused with

stimulated neutrophils, but not when neutrophils from patients with CGD were used (Shasby et al., 1982b).

The normal lung contains a 'marginated' pool of neutrophils in a state of dynamic equilibrium with the circulating pool; this localisation of potentially destructive cells may explain why the lung is at risk of injury from a variety of local and distant insults. Inflammatory mediators, particularly bacterial endotoxin (LPS), reduce neutrophil deformability and enhance pulmonary vascular sequestration of neutrophils (Haslett et al., 1987), and trace amounts of LPS synergise with neutrophil chemotactic factors to damage rabbit pulmonary vascular endothelium (Worthen et al., 1987). Circulating endotoxin has been associated with the development of ARDS in humans (Parsons et al., 1989). These factors have led to a proposed mechanism for the development of ARDS (Haslett and Donnelly, 1992); inflammatory mediators promote pulmonary sequestration of neutrophils by reducing deformability and enhancing adhesion receptor expression (see Chapter 4), and prime them for enhanced release of oxygen radicals and proteases (see Section 1.7) in a restricted neutrophil-endothelial environment which favours cell injury.

Despite all the evidence implicating the neutrophil as a key mediator of tissue injury in ARDS, the syndrome has been reported to occur in granulocytopenic patients (Braude et al., 1985), suggesting that neutrophil-independent mechanisms (for example, infection, graft-versus-host disease and involvement of other cell types such as monocytes/macrophages) can also lead to ARDS. The fact that ARDS is encountered very infrequently in neutropenic patients who are prone to recurrent severe gram-negative infections again suggests that neutropenia may protect against ARDS to a certain extent. In a study of 14 patients with chemotherapy-induced neutropenia and ARDS, Rinaldo and Borovetz (1985) noted that the gas-

exchange abnormalities worsened as the peripheral neutrophil counts increased towards normal, providing evidence that neutrophils are an important, but not the sole cause of lung injury in patients with ARDS.

1.7. Neutrophil Priming

Neutrophils do not express their full microbicidal effector functions (generation of ROI and bioactive lipids, degranulation, etc.) in response to biological activating agents unless they have first been primed. Priming refers to the process whereby the response of a cell to a subsequent (activating) stimulus is potentiated, sometimes greatly, by previous exposure to a priming agent. The priming agent does not by itself initiate the effector function(s) and must be presented to the cell before the activating stimulus.

Neutrophil priming was first demonstrated in 1984 by Guthrie et al., who showed that preincubation of freshly isolated human neutrophils with bacterial endotoxin did not in itself elicit a respiratory burst, but greatly enhanced the oxidative response to fMLP, and to a far lesser extent to PMA. fMLP applied to unprimed neutrophils stimulated only minimal O_2^- generation: indeed, since the manipulations required to isolate neutrophils themselves may cause priming (Haslett et al., 1985) truly unprimed circulating neutrophils may be totally unresponsive to fMLP-induced degranulation or O_2^- generation unless they are exposed to priming agents.

A wide array of substances, both physiological and physicochemical, have since been shown to act as priming agents (see Table 1.1). The variety of preincubation times required to obtain a maximally primed response, which ranges from a few seconds (ATP) to several hours (LPS, GM-CSF, interferon- γ), implies that differing signal transduction routes are utilised by these agents. In addition to the respiratory burst, priming of the degranulation response (Fittschen et al., 1988) and of the generation of lipid

Figure 1.3

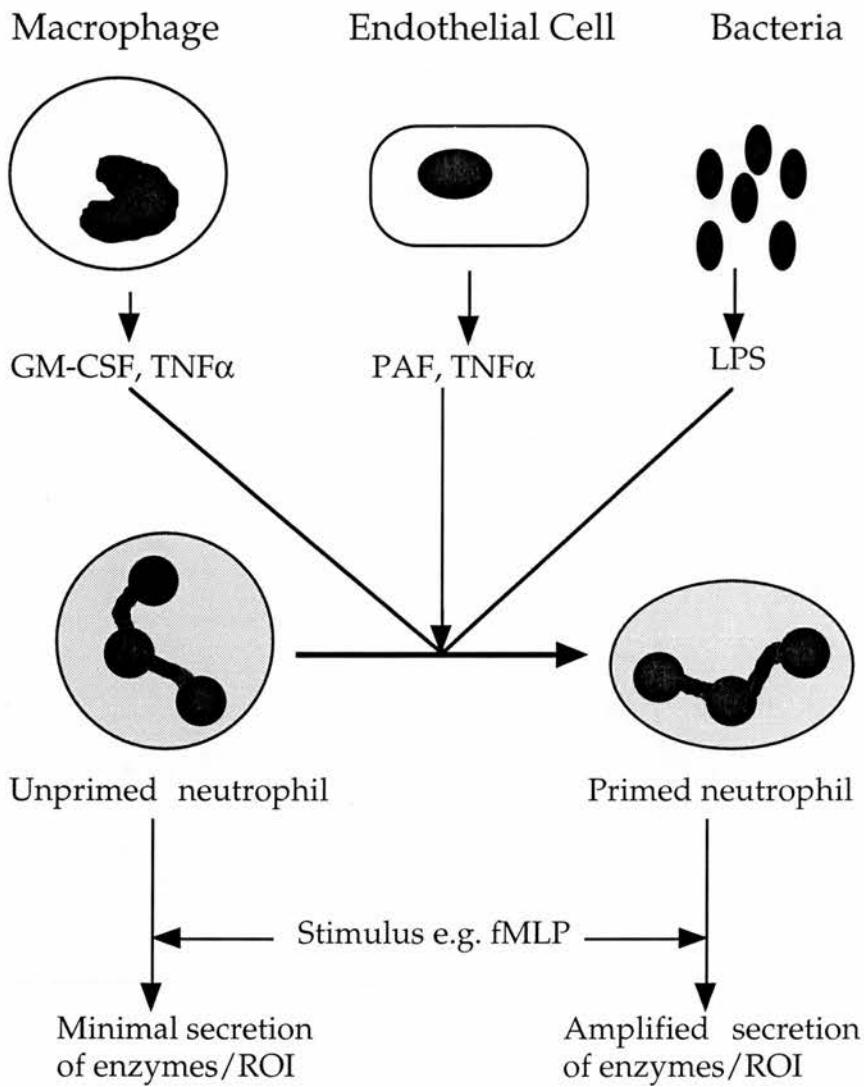


Figure 1.3 Neutrophil Priming

Priming of neutrophils by the products of activated macrophages (e.g. $\text{TNF}\alpha$, GM-CSF), endothelial cells (e.g. PAF) and bacteria (e.g. LPS) does not lead directly to the release of toxic products but results in amplified secretion of reactive oxygen intermediates and enzymes when a further stimulus (eg. fMLP) is encountered.

Table 1.1

Priming Agent	Time for Maximal Priming	Reference
ATP	15 s	Kuhns et al., 1988
Substance P	1 min	Lloyds et al., 1993
Ionomycin	2 min	Finkel et al., 1987
Inositol hexakisphosphate	2 min	Eggleton et al., 1991
L-selectin cross-linking	3 min	Waddell et al., 1994
PAF	5 min	Vercellotti et al., 1988
CD18 cross-linking	5 min	Liles et al., 1995
TNF α	10 min	Berkow et al., 1987
Interleukin-8	10 min	Daniels et al., 1992
Orthovanadate	10 min	Lloyds et al., 1994
Influenza A virus	30 min	Busse et al., 1991
LPS	120 min	Guthrie et al., 1984
GM-CSF	120 min	Weisbart et al., 1986a
Interferon- γ	120 min	Tennenberg et al., 1993

mediators (principally arachidonic acid (AA), leukotriene B₄ (LTB₄) and PAF; Doerfler et al., 1989 and 1994) has been described. Different mediators have also been reported to display a different spectrum of priming activity; for example, unlike LPS, GM-CSF primes the oxidative burst stimulated by fMLP but not by PMA (Weisbart et al., 1986a). Additionally, whilst TNF α produces up to a 20-fold augmentation of the fMLP-stimulated respiratory burst, weaker priming agents exist, such as inositol hexakisphosphate (InsP₆; Eggleton et al., 1991) and interferon- γ

(Tennenberg et al., 1993) that elicit at best a doubling of the response. A final complication is that sub-threshold concentrations of classical 'activating' agents such as fMLP can act as primers (English et al., 1981). Any mechanism proposed to underlie priming must encompass these facts.

1.7.1 Neutrophil Priming in Vivo

Although priming was described initially as an *in vitro* phenomenon, many priming agents are biologically relevant inflammatory mediators released in response to infection, trauma, haemorrhage and inflammatory states. Circulating endotoxin has been associated with the development of ARDS (Parsons et al., 1989), and persisting high levels of TNF α and interleukin-6 (IL-6) have been linked to poor outcome in septic shock (Pinsky et al., 1993). While cytokines are detectable in the bloodstream only in extreme circumstances, locally generated mediators will upregulate the functional responses of extravasated neutrophils; indeed, since cross-linking of neutrophil adhesion receptors is itself a priming stimulus (Waddell et al., 1994, Liles et al., 1995), the process of extravasation *per se* may result in a degree of priming. Additionally, while most studies have concentrated on isolated agents, a few studies have reported synergy between priming agents (Elbim et al., 1994, Roberts et al., 1993), a situation which is likely to be more analogous to the cytokine cascade generated by an inflammatory response. Two approaches have been used to study priming *in vivo*; firstly, investigators have infused priming agents into laboratory animals or human volunteers and studied granulocyte responses, and secondly, neutrophils isolated from patients suffering from infection/inflammation have been functionally assessed.

Continuous infusion of endotoxin into rats was found to prime the respiratory burst of neutrophils isolated from the liver of the subjects at 3

hours and to a lesser extent at 30 hours (Mayer and Spitzer, 1991). Intravenous injection of TNF α into healthy human volunteers resulted in neutrophil activation with evidence of circulating elastase and lactoferrin (Van der Poll et al., 1992) or in priming of circulating neutrophils for release of hypochlorous acid (Wewers et al., 1990). Primed neutrophils have been identified in the peripheral blood of patients following blunt trauma (Krause et al., 1988), ARDS (Chollet-Martin et al., 1992) and bacterial (Gram-positive and Gram-negative) and fungal infection (Bass et al., 1986) and from the joints of patients with active rheumatoid arthritis (Robinson et al., 1992). Because of the diversity of the signalling processes that they utilise, and because of their physiological importance as inflammatory mediators, the priming of human neutrophils by LPS, TNF α and PAF has been studied in detail in the work presented in this thesis. These agents will be discussed in greater detail below, followed by consideration of the pathways potentially used to transduce the priming signal.

1.7.2 LPS as a Priming Agent

LPS, a complex glycolipid, is the major component of the outer membrane of Gram-negative bacteria and is released when bacteria die/lyse and when they multiply. Structurally, LPS consists of a variable polysaccharide domain covalently bonded to a highly conserved diglucosamine-based phospholipid, lipid A. Purified lipid A has potent LPS-like agonist activity, suggesting that it is responsible for most, if not all, of the biological effects of endotoxin (Galanos et al., 1985). The clinical syndrome of Gram-negative septicaemia appears to result primarily from excessive stimulation of the host immune response by LPS. LPS-induced activation of macrophages results in the production of cytokines such as TNF α and interleukins -1, -6, -8 and -10, which mediate the fever, hypotension and other features of septic

shock (Galanos and Freudentberg, 1993). In addition to its effects on macrophages, endotoxin initiates sequestration of neutrophils in the pulmonary vasculature; together with its priming activity this may predispose the pulmonary vascular endothelium to neutrophil-mediated injury (Haslett et al., 1987).

The initial experiments of Guthrie et al. (1984) required fairly high concentrations of LPS (≥ 10 ng/ml) to prime neutrophils; Aida and Pabst (1990) demonstrated that neutrophils washed free of plasma responded only weakly to LPS, but were primed by as little as 1 ng/ml in the presence of plasma. The component of plasma required for optimal LPS activity was identified as lipopolysaccharide binding protein (LBP: Vosbeck et al., 1990), an acute phase protein synthesised by the liver. The formation of complexes between LPS with LBP not only allowed priming by lower concentrations of LPS but also accelerated the process to the extent that a maximal response could be achieved within a 30 min preincubation of cells with LPS-LBP.

Priming of neutrophils by LPS can be abrogated by antibodies to CD14, a GPI-linked membrane glycoprotein thought to act as the receptor for the LPS-LBP complex (Weingarten et al., 1993, Shapira et al., 1995); in addition, CD14 is present in secretory granule membranes, forming a cellular pool that can be rapidly deployed to the cell surface in response to degranulating stimuli (Detmers et al., 1995). Since CD14 lacks a cytoplasmic domain, the mechanism by which signal transduction occurs is poorly understood. The β_2 integrin, CD11c/CD18, has also been proposed to act as an LPS receptor, since transfection of this molecule into CHO cells (which lack both the integrin and CD14) confers serum-independent responsiveness to LPS to these previously unresponsive cells (Ingalls et al., 1995).

Figure 1.4

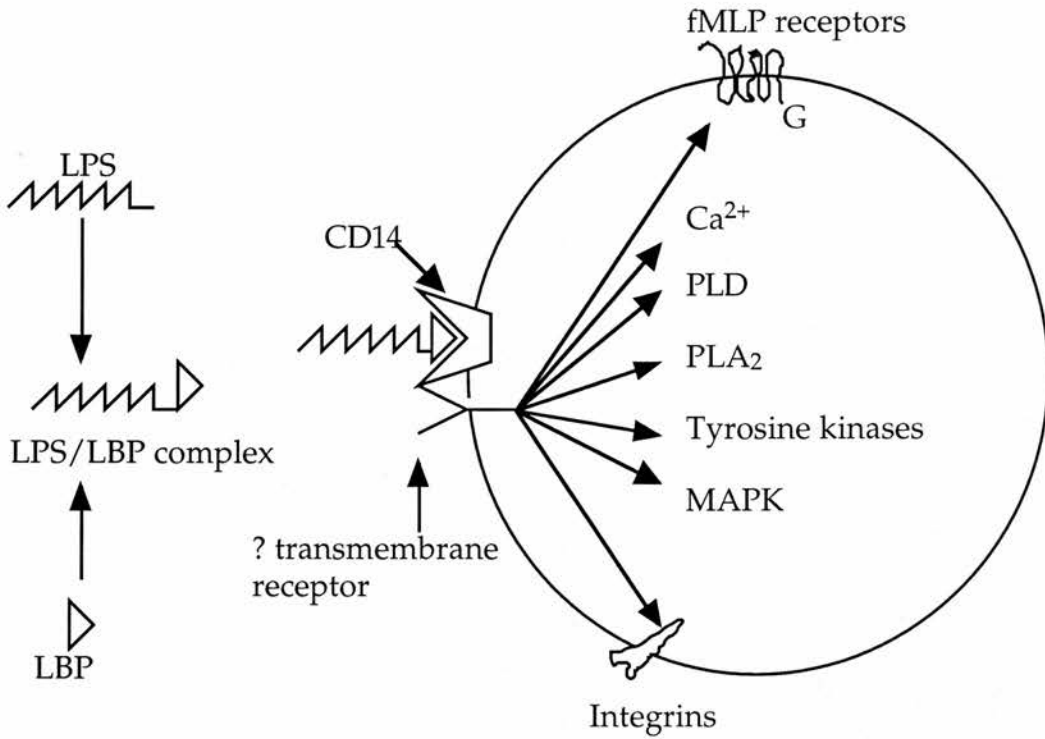


Figure 1.4 Neutrophil Priming by LPS

LPS complexed with LBP interacts with neutrophil CD14; possibly acting via an as yet unidentified transmembrane receptor, this may result in upregulation of fMLP receptors and integrins, and the activation of several signalling pathways (see section 1.8)

1.7.3 $\text{TNF}\alpha$ as a Priming Agent

$\text{TNF}\alpha$ was originally described as a cytokine capable of inducing tumour necrosis (Carswell et al., 1975) but was subsequently discovered to modulate the immune system. It is produced principally by monocytes and macrophages in response to LPS, but also by lymphocytes, fibroblasts and even by neutrophils themselves (Dubravec et al., 1990). $\text{TNF}\alpha$ mimics many

of the effects of endotoxin when injected into experimental animals or human volunteers (Tracey et al., 1986). It is a potent neutrophil priming agent, upregulating agonist-induced O_2^- by up to 20-fold (Berkow et al., 1987). It was therefore initially proposed that LPS priming was mediated by $TNF\alpha$ secreted by small numbers of contaminating monocytes in neutrophil preparations; however, Aida and Pabst (1990) demonstrated failure of anti- $TNF\alpha$ antibodies to inhibit the priming effects of LPS.

Figure 1.5

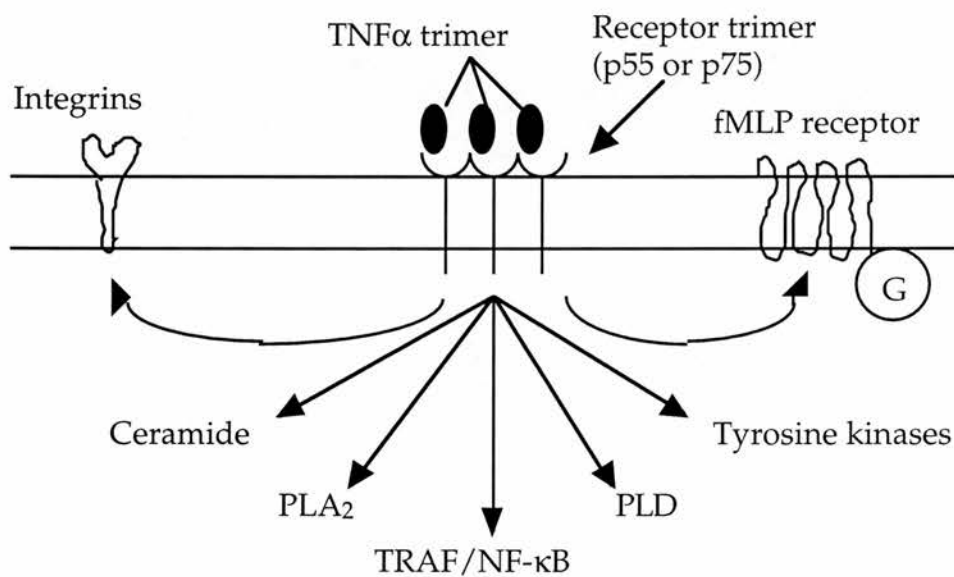


Figure 1.5 Neutrophil Priming by $TNF\alpha$

Interaction with trimeric $TNF\alpha$ leads to receptor clustering and activation of various signal transduction processes, with up-regulation of fMLP receptors and adhesion molecules. How these changes may lead to the establishment of the primed state is discussed in 1.8.

Mature TNF α is a homotrimer composed of 17 kDa subunits, which interact with 2 distinct receptors of 55 and 75 kDa (TNF-R55/CD120a and TNF-R75/CD120b; Brockhaus et al., 1990) inducing receptor clustering. Signalling pathways activated by the interaction of TNF α with its receptors include the sphingomyelinase-ceramide pathway (see 1.8.10) and the recently described TNF receptor associated factor (TRAF) proteins (Rothe et al., 1995) which mediate activation of the transcription factor NF- κ B.

1.7.4 PAF as a Priming Agent

The chemical structure of PAF (1-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) was elucidated in 1979 by, among others, Demopoulos et al. (1979); in addition to platelets, neutrophils, mononuclear cells, eosinophils and liver cells are all responsive to this lipid mediator (Chao and Olson, 1993). The initial rate-limiting step in its production is the hydrolysis of arachidonate-containing membrane phospholipids by phospholipase A₂ to yield arachidonic acid and lyso-PAF (Chilton et al., 1984); activation of phospholipase A₂ requires both phosphorylation of the enzyme, and a calcium-dependent translocation from cytosol to membrane (Lin et al., 1993). Lyso-PAF is converted to PAF by Ca²⁺/calmodulin-dependent acetyltransferase, and PAF interacts with a G-protein-linked, 7-transmembrane receptor, which has been cloned (Nakamura et al., 1991).

PAF does not stimulate the respiratory burst directly except at very high concentrations, but is a potent priming agent (Vercellotti et al., 1988), efficacious after brief preincubations (<1 min). Endothelial monolayers treated with thrombin prime neutrophils layered on top by the production of PAF, suggesting that this is a biologically relevant priming effect (Vercellotti et al., 1989). In an in vivo study of endotoxic shock, elevated plasma levels of both TNF α and PAF were required to produce severe hypotension (Sun et

al., 1990), further emphasising the *in vivo* significance of PAF as a biological mediator.

1.8 Signalling Pathways in Neutrophil Priming

Since the respiratory burst provoked by fMLP is the most readily 'primable' neutrophil function, it is also the most studied. However, other neutrophil functions can also be primed (degranulation: Haslett et al., 1985, Fittschen et al., 1988, and the synthesis of bioactive lipids including PAF and leukotriene B₄, Doerfler et al., 1989), and other stimuli may activate primed neutrophils (e.g. opsonised zymosan, PAF, PMA etc.). Studies concentrating on one system cannot automatically be extrapolated to all priming situations. Since priming and activation are so closely linked, and since most priming agents can activate neutrophils either when applied in high concentrations or when applied to adherent neutrophils, most investigators have concentrated on signalling mechanisms known to be involved in the generation of oxidative burst activity.

1.8.1 Modulation of Agonist Receptors

Upregulation of the number and/or affinity of agonist receptors could augment subsequent responses to the same agonist. fMLP and other chemoattractants bind to G-protein-coupled receptors containing seven membrane-spanning segments. The effects of priming agents on fMLP receptors have been studied by several authors, with conflicting results (Table 1.2).

Such results need to be interpreted with caution. O'Flaherty et al. (1991) demonstrated that TNF α increased fMLP binding to neutrophils, but this effect lagged behind the cytokine's priming of fMLP-induced degranulation

Table 1.2

Priming agent	Effect on fMLP Receptors	Reference
TNF α	Non-significant increase	Berkow et al., 1987
TNF α	Increased (but dissociated from priming)	O'Flaherty et al., 1991
LPS	Non-significant decrease	Guthrie et al., 1984
LPS	2-3-fold increase	Vosbeck et al, 1990
LPS	3-fold increase	Howard et al., 1990
GM-CSF	Increased numbers Decreased affinity	Weisbart et al., 1986b
GM-CSF, IL-8	Increased	Roberts et al., 1993
IFN- γ	None	Tennenberg et al., 1993

and hence could not be responsible for priming. In the study by Roberts et al., (1993) priming of the respiratory burst by IL-8 was maximal at 10-50 ng/ml, but no increase in fMLP receptor number was detectable at concentrations below 100 ng/ml. Berkow et al. (1987) noted that TNF α induced a 30% increase in fMLP receptor number, which did not reach statistical significance; the associated priming of the oxidative response was 280%. Thus effects on fMLP receptors can be dissociated from priming. Neutrophils possess granule-associated fMLP receptors (section 1.3) that are mobilised to the cell surface on degranulation, and the presence of increased surface fMLP-binding may reflect exocytosis rather than priming.

Few investigators have quantified the effects of priming on other chemoattractant/agonist receptors; however, the study by O'Flaherty et al. (1991) revealed that TNF α -priming of the degranulation response to PAF and LTB₄ was associated with a reduction in surface LTB₄ receptors and only a transient increase in PAF receptors, again arguing against a critical role for receptor modulation in priming.

1.8.2 Heterotrimeric GTP-binding Proteins

As stated above, chemoattractant receptors are coupled to heterotrimeric G-proteins, which consist of α - and $\beta\gamma$ -subunits. Agonist-binding induces a conformational change in the receptor, activating the G-protein by allowing GTP to replace GDP bound to the α -subunit; the subunits dissociate and are free to interact with downstream effectors (Neer and Clapham, 1988; see Figure 1.6). The intrinsic GTP-ase activity of the α -subunit hydrolyses GTP to GDP, and α -GDP recombines with free $\beta\gamma$, ending the activation cycle. Whilst many different subunits have been identified, in neutrophils the predominant α -subunit is G_{i α 2} (Goldsmith et al., 1987); the closely related G_{i α 3} (Itoh et al., 1988) is also present. The identity of the $\beta\gamma$ subunits, and the exact identity of the G-protein(s) coupling the fMLP receptor to its various effectors are unknown.

Both G_{i α 2} and G_{i α 3} are substrates for pertussis toxin, which ADP-ribosylates and so inactivates them. Pertussis toxin abolishes superoxide production induced by fMLP, making it impossible to use the toxin to study priming of this response. However, responses to PMA or arachidonic acid are not suppressed; Berkow and Dodson (1988) demonstrated that the slight priming effect of TNF α on PMA-induced O₂⁻ production was not abolished by pertussis toxin. Corey and Rossoff (1989) reported that pertussis toxin

Figure 1.6

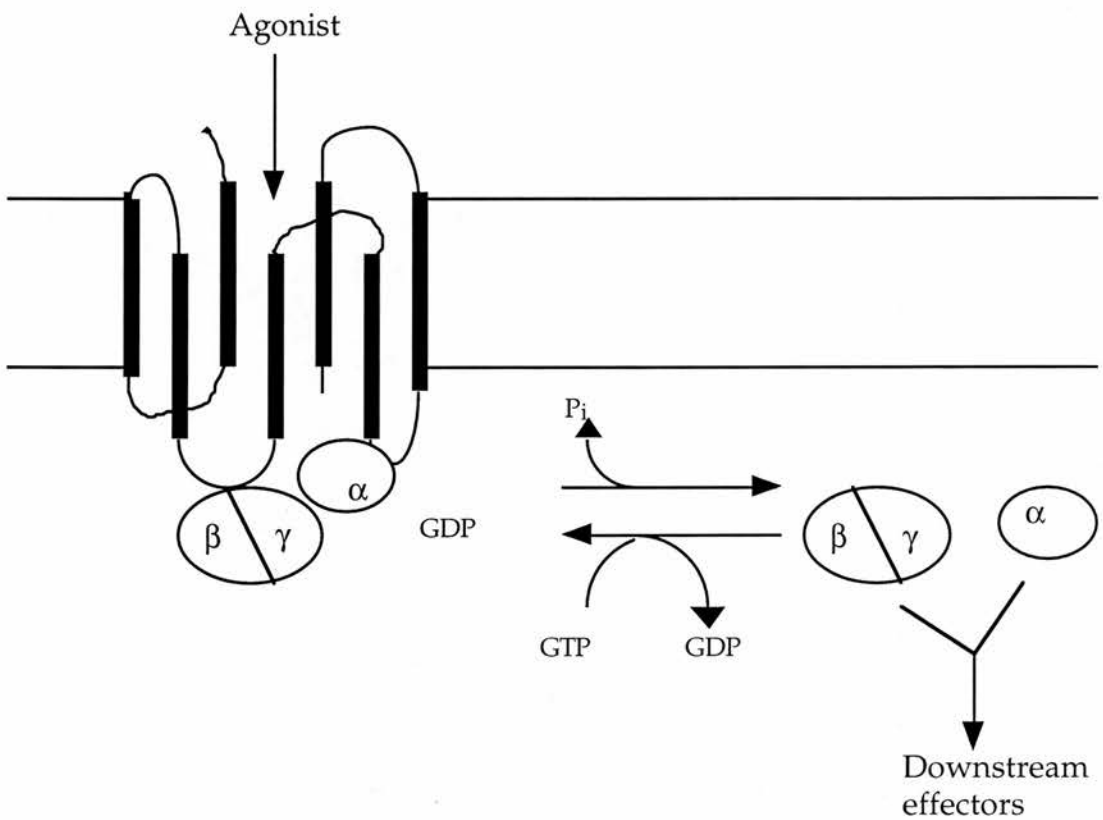


Figure 1.6 Signalling by G-protein-linked Receptors

Binding of agonist to the pocket formed by the 7 transmembrane components of the receptor induces a conformational change, altering the interaction with the associated G-protein. As a result, GTP replaces the GDP bound to the α -subunit, and the subunits dissociate to interact with downstream effectors. The intrinsic GTP-ase activity of the α -subunit hydrolyses GTP to GDP, releasing inorganic phosphate, and the G-protein is reconstituted from the free individual components.

abolished GM-CSF priming of arachidonic acid-induced oxidative activity, whilst DiPersio et al. (1988) observed no effect on the same process. Clearly pertussis toxin is not an ideal tool for the investigation of the role of G-proteins in priming.

For G-proteins to interact with receptors, they must be present at the plasma membrane. A few groups have investigated the effects of priming agents on G-protein translocation and function. A study by Klein et al. (1992) demonstrated enhanced membrane G-protein expression in HL-60 cells (a leukaemia cell line) following incubation with interferon- γ ; however, a 24 hour pretreatment was required which does not correlate with priming. Yasui et al. (1992) demonstrated that LPS 10 ng/ml in the presence of 1% serum (45 min, 37°C) increased the amount of $G_{i\alpha 2}$ present in neutrophil membranes; other concentrations and incubation times were not studied, but these incubation conditions were shown to prime the respiratory burst. Durstin et al. (1993) showed that incubation with GM-CSF induced translocation of $G_{i\alpha 2}$ from granule membranes to the neutrophil plasma membrane; this was significant at 5 min and maximal by 40 min; no effect on $G_{i\alpha 3}$ was found. No priming data was presented in this second study; it should be noted that maximal priming with GM-CSF has been demonstrated (Weisbart et al., 1986) to require 2 hours. Klein et al. (1995) demonstrated that TNF α (100 U/ml, 10 min) increased the amount of fMLP receptor-associated $G_{i\alpha 2}$ and $G_{i\alpha 3}$ in the neutrophil membrane and confirmed enhanced G-protein activity on fMLP-stimulation. Taken together, these data suggest that a translocation of G-proteins to the neutrophil membrane might contribute to neutrophil priming, but more detailed analyses are required.

1.8.3 Phospholipase C Activation

The β_2 isoform of PIC is activated by dissociated G-protein $\beta\gamma$ -subunits (Katz et al., 1992) and the β_1 isoform by α -subunits (Rhee and Choi, 1992) to hydrolyse its membrane phospholipid substrate $\text{PtdIns}(4,5)\text{P}_2$. This reaction generates $\text{Ins}(1,4,5)\text{P}_3$ (the signal for release of Ca^{2+} from intracellular stores) and DAG, an activator of protein kinase C; DAG can also be generated, often in a more sustained fashion, by the action of phospholipase D (see below, 1.8.4). The PIC- γ isoform is activated by tyrosine phosphorylation (Rhee, 1991) but the functional significance of this isoform in neutrophils is unclear at present.

1.8.3.1 $\text{Ins}(1,4,5)\text{P}_3$ Accumulation

The application of fMLP to a neutrophil population results in a rapid but transient elevation of $\text{Ins}(1,4,5)\text{P}_3$, with a peak at 10-15 sec and thereafter a rapid return to basal levels. Despite a clear demonstration that the resultant calcium transient is an essential ingredient in activation of the respiratory burst (Dewald et al., 1988), remarkably few studies have focused on the potential for this second messenger pathway to be upregulated in the priming process. Both unchanged (Corey and Rosoff 1989, Bourgoignie et al., 1990) and increased (Macphie, 1992) resting and stimulated $\text{Ins}(1,4,5)\text{P}_3$ levels have been reported in GM-CSF-primed cells. Grimminger et al. (1991) reported that botulinum C_2 toxin (which ADP-ribosylates G-actin, preventing actin filament assembly) primed neutrophils for superoxide production, and enhanced total inositol phosphate accumulation in response to PAF, fMLP and LTB_4 , but stated that the increase was exclusively attributable to increased InsP_1 accumulation, with no effect on $\text{Ins}(1,4,5)\text{P}_3$. All of these studies utilised cells which had undergone lengthy (> 2 h) labelling with [^3H] inositol to enable quantification of the separate inositol

phosphates; such procedures could affect neutrophil responses, and no studies have employed the recently developed mass assay (Challiss et al., 1988) to measure Ins(1,4,5)P₃ accumulation.

1.8.3.2 Elevation of [Ca²⁺]_i

Binding of Ins(1,4,5)P₃ to its intracellular receptor induces a conformational change and results in efflux of Ca²⁺ from intracellular stores; emptying of the stores may activate influx of Ca²⁺ from the external medium (capacitative Ca²⁺ entry: Putney, 1986). fMLP, PAF and other agonists induce PtdIns(4,5)P₂ hydrolysis and hence cause elevations in the [Ca²⁺]_i. Neutrophils depleted of Ca²⁺ do not undergo a respiratory burst (Dewald et al., 1988), and Hallett et al. (1990) identified a threshold [Ca²⁺]_i of 250 nM; the oxidase of individual neutrophils always became activated when this threshold was exceeded. Whilst it is generally accepted that a rise in [Ca²⁺]_i is an essential step in activation, its role in priming is less clear. Calcium ionophores such as ionomycin act as priming agents, and Finkel et al. (1987) correlated the elevation of [Ca²⁺]_i with the degree of priming in response to ionomycin. Priming agents may act by recruiting more cells into a 'calcium responsive' population (Elsner et al., 1992). However, as can be seen from Table 1.3, there are conflicting reports on the effects of individual priming agents on resting and stimulated [Ca²⁺]_i. It is generally agreed that TNF α does not mobilise Ca²⁺ or augment the Ca²⁺ transient generated in response to other agonists, and similar results have been reported for other agents such as substance P. Despite consistent reports that PAF mobilises Ca²⁺ and may augment the stimulated transient, Koenderman et al. (1989) reported that PAF-induced priming was only partially inhibited under [Ca²⁺]_i-buffering conditions, and these observations were confirmed by Walker et al. (1991). Finally, in experiments by Wymann et al. (1987) application of fMLP

Table 1.3

Priming Agent/ Ca ²⁺ Indicator	[Ca ²⁺] _i		Reference
	(Basal)	(Stimulated)	
LPS/fura2	↑	↑	Forehand et al., 1989
LPS/fura2	→	→	Doerfler et al., 1989
LPS/fluoro-3	↑	↑	Yee and Christou, 1993
LPS/fura2	→	not done	Klein et al., 1990
TNFα/quin2	→	→	Yuo et al., 1989
TNFα/fura2	→	→	Lloyds et al., 1995
PAF/fura2	→	↑	Vercellotti et al., 1988
PAF/fura2	↑	↑	Worthen et al., 1988
GM-CSF/quin2	→	→	Sullivan et al., 1987
GM-CSF/fura2	↑	↑	Naccache et al., 1988
GM-CSF/fura2	→	↑	McColl et al., 1991
Substance P/fura2	→	→	Lloyds et al., 1993
ATP/quin2	↑	→	Kuhns et al., 1988
Orthovanadate/ fura2	→	→	Lloyds et al., 1994

2 min after a low concentration of PMA (1.6 nM) resulted in enhanced chemiluminescence approximately 2 s before a rise in fura-2 fluorescence, suggesting that priming of the oxidative burst does not rely on enhanced calcium fluxes. Taken together, these results seem to suggest that neither

increased baseline nor stimulated $[Ca^{2+}]_i$ are essential for priming, although such effects may contribute to the priming mechanism of some agents.

1.8.3.3 DAG and Protein Kinase C

Phorbol esters such as PMA bind to and activate the classical serine/threonine kinase protein kinase C (cPKC) family of enzymes (Castagna et al., 1982), inducing phosphorylation of several substrates including p47^{phox} and activating the NADPH oxidase. PKC translocates to the cell membrane within seconds of stimulation by agents such as fMLP (Christiansen, 1988); at the membrane, DAG produced by the actions of phospholipase C and/or D, in conjunction with the membrane phospholipid phosphatidylserine, activate the kinase (Lee and Bell, 1991). Synthetic DAGs and phorbol esters activate the respiratory burst suggesting that PKC is involved in at least one of the activation pathways (Robinson et al., 1985). Neutrophils have been shown to possess at least two PKC isoforms, the calcium-dependent β_1 -PKC (the most abundant isoform) and the calcium-independent η -PKC (Majumdar et al., 1991 and 1993). PMA in sub-activating concentrations primes neutrophils, indicating a possible role for PKC in the priming process.

The effects of priming agents on the PKC-activator DAG are somewhat controversial. Tyagi et al. (1989) reported that GM-CSF (100 pM) alone produced only a small increase in neutrophil DAG mass but markedly increased DAG accumulation in response to fMLP, and Bourgoin et al. (1990) also described an augmented DAG response to the chemotactic peptide in GM-CSF-primed neutrophils. In contrast, Bauldry et al. (1991a) found that treatment of polymorphonuclear cells with TNF α did not induce DAG formation, and that in the absence of cytochalasin B there was no enhancement of the response to fMLP in TNF α -treated cells.

Whilst some studies have suggested that priming may enhance DAG generation, several studies have failed to show translocation/activation of PKC in primed cells; hence LPS (Forehand et al., 1989), TNF α (Berkow and Dodson, 1988) and GM-CSF (Sullivan et al., 1987) all failed to induce PKC translocation under priming conditions. Despite these findings, Thelen et al. (1990) demonstrated enhanced phosphorylation of the myristolated, alanine-rich C kinase substrate (MARCKS) in cells primed with LPS or TNF α ; however, this action was delayed with respect to priming, and the enhancement was blocked by cycloheximide (priming by these agents is not dependent on protein synthesis, see 1.8.10 below). The increased detection of the phosphorylated MARCKS was attributed to LPS- and TNF α -induced synthesis of the kinase substrate rather than to increased kinase activity, and is unlikely to be relevant to priming. Thus the weight of evidence is against augmented PKC activation playing a significant role in priming.

1.8.4 Phospholipase D Activation

Phospholipase D catalyses the hydrolytic cleavage of the terminal phosphodiester bond of PC or PtdIns(Px) to yield phosphatidic acid (PA) and choline or Ins(Px); fMLP-mediated stimulation of PC-specific PLD in neutrophils was demonstrated by Cockcroft (1984). PA is converted to DAG by phosphatidate phosphohydrolase (Billah et al., 1989), and this results in a second, usually more sustained phase of DAG generation than that resulting from the activation of PIC (Truett et al., 1988, Billah et al., 1989). The role of DAG in activating PKC has been discussed (1.8.3.3); PA is involved in activation of the NADPH oxidase (Bonser et al., 1989, Morel et al., 1991) and in changes in the actin cytoskeleton (Ha and Exton, 1993).

Bourgoin et al. (1990) and Bauldry et al. (1991a) studied the effects of priming (with GM-CSF and TNF α , respectively) on phospholipase D

activity; both demonstrated that while treatment of cells with the priming agent alone did not result in accumulation of PA, the subsequent fMLP-induced accumulation was augmented and prolonged in primed cells. In the latter study, PA production was found to correlate closely with the amount of O_2^- generation that occurred, suggesting that enhancement of PLD activity could play a role in priming.

1.8.5 Phospholipase A₂ Activation

Phospholipase A₂ releases free AA from cellular phospholipids (Walsh et al., 1983) in what is believed to be the rate-limiting step in the synthesis of biologically active eicosanoids (Van den Bosch, 1980). Priming agents can enhance the synthesis of bioactive lipids (AA, PAF, LTB₄) in response to activating stimuli (Bauldry et al., 1991b, DiPersio et al., 1988, Doerfler et al., 1989); additionally, activation/translocation of PLA₂ has been suggested as a potential mechanism of priming. Smith and Waite (1992) demonstrated that a PLA₂ activity was translocated from the cytosolic fraction to the plasma-membrane fraction in LPS-primed neutrophils; Doerfler et al. (1994) confirmed this finding and in addition demonstrated that LPS induced the phosphorylation of PLA₂, an event previously correlated with PLA₂ activation (Lin et al., 1993). Fouda et al. (1995) also demonstrated that LPS induced the phosphorylation of PLA₂, and detected a small but significant release of arachidonic acid by LPS alone in addition to its priming effect. Enhanced AA availability was proposed to explain the priming by GM-CSF of IL-8-stimulated lipid mediator release, since IL-8 itself lacks the ability to promote the release of endogenous AA (McDonald et al., 1993). However LPS-priming of LTB₄ release was noted only in response to PMA, calcium ionophore or opsonised zymosan, and not to fMLP (Doerfler et al., 1989), and none of the above studies have linked PLA₂ activation with priming of

the respiratory burst. Worthen et al. (1988) correlated LPS-induced synthesis of intracellular PAF with priming of the respiratory burst, and suggested that PAF could be responsible for the priming of the respiratory burst by LPS; however, Stewart et al. (1991) demonstrated that neither blockade of PAF receptors (WEB 2086) nor inhibition of LTB₄ synthesis (CGS8515) influenced the priming of fMLP-mediated O₂⁻ generation by TNFα or GM-CSF. Finally, Ely et al. (1994) showed that TNFα-primed release of AA and O₂⁻ are independent events, since when released under physiological conditions AA was unable to stimulate or modulate O₂⁻ release. It therefore seems unlikely that PLA₂ is responsible for the priming of the respiratory burst, although it may be an important messenger for enhanced lipid mediator release.

1.8.6 Neutrophil Adhesion Molecules and the Cytoskeleton

Intravascular sequestration of neutrophils at inflamed sites and migration through the vascular endothelium depends on the sequential regulation of expression and function of the cell adhesion molecules (members of the immunoglobulin superfamily, integrins and selectins; see Chapter 4). Once in the extracellular environment, neutrophils interact with extracellular matrix proteins predominantly through cell surface integrins. These receptors interact with the cytoskeleton, integrating the extracellular milieu with the cell interior.

The vascular selectins (CD62-P/P-selectin and CD62-E/E-selectin) and the constitutively-expressed leukocyte antigen CD62-L (L-selectin) mediate the initial, low affinity 'rolling' interaction between the neutrophil and the vascular endothelium (Bevilacqua and Nelson, 1993, Carlos and Harlan, 1994, Albelda et al., 1994). While low concentrations of many secretagogue agonists have been shown to induce loss of CD62-L from the neutrophil

surface (Kishimoto et al., 1989, Griffin et al., 1990), this event has not been studied in parallel with neutrophil priming. Cross-linking of CD62-L by sulfatides or by monoclonal antibodies has, however been shown to prime neutrophils (Waddell et al., 1994), suggesting a close link between the processes of transmigration and priming.

Firm adhesion is principally a function of the leukocyte β_2 -integrins, particularly CD11b/CD18 (Mac-1), whose expression and function are upregulated by a number of secretagogue agonists (Smith et al., 1989). Cross-linking of the CD18 integrin component with monoclonal antibodies has also been demonstrated to lead to neutrophil priming (Liles et al., 1995), although again, upregulation of integrins has not been correlated directly with the establishment of the primed state. Activation of respiratory burst activity by TNF α has been shown to be dependent on the engagement of neutrophil integrins (Nathan et al., 1989a). Interaction of integrins with ligand (ICAM-1) results in receptor clustering and activation of signalling pathways (Rosales and Juliano, 1995) including Ca^{2+} transients, phosphorylation of the focal adhesion kinase (p125^{FAK}) which is involved in cytoskeletal organisation, stimulation of the Na^+/H^+ antiport, and possibly activation of the mitogen activated protein kinase (MAPK - see 1.8.7). Thus agonists which regulate neutrophil adhesion molecules may also influence the hyperresponsive state of the primed neutrophil (Figure 1.7).

In resting blood neutrophils, only about 20-30% of the cellular actin is in the polymerised form, and most of this is present in a filamentous network adjacent to the plasma membrane, with which it can interact via integral membrane proteins (Sheterline et al., 1984). Stimulation results in the rapid assembly of actin polymer (F-actin) from the monomeric G-actin pool, particularly in developing lamellopodia (Wallace et al., 1984). fMLP-induced

Figure 1.7

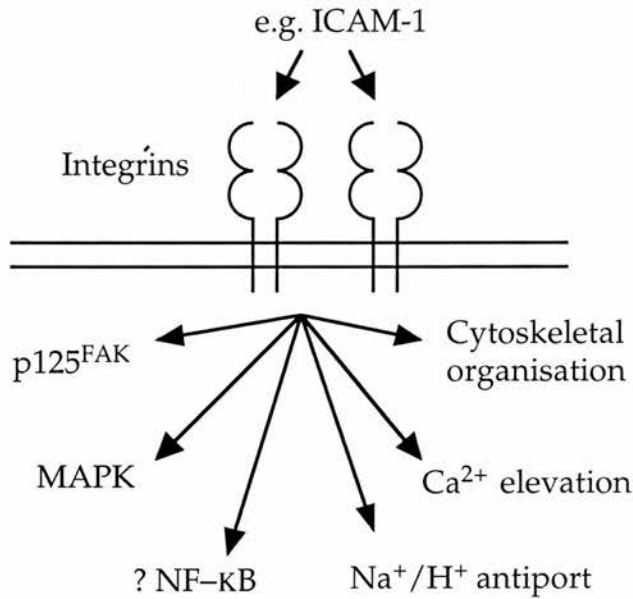


Figure 1.7 Signalling by Integrins

ICAM-1-integrin interaction results in clustering of integrin molecules and activates a series of signal transduction sequences. Changes in Ca^{2+} , pH (via the activation of the Na^+/H^+ antiport), phosphorylation (of p125^{FAK} and MAPK) and gene induction (in monocytes) have all been observed after integrin engagement.

polymerisation has been shown to be enhanced by co-incubation with IL-8 and other cytokines (Brom et al., 1991), by pre-incubation with InsP_6 (Crawford and Eggleton, 1992) and by preincubation with LPS (Howard et al., 1990). However, other studies have refuted the link between actin polymerisation and priming; for example, Lloyds and Hallett (1993) demonstrated that activating, but not priming concentrations of substance P induced actin polymerisation directly (the effect on fMLP-induced polymerisation was not documented) and Grimminger et al. (1991)

demonstrated that treatment of neutrophils with botulinum C₂ toxin (which ADP-ribosylates G-actin) suppressed fMLP-initiated actin polymerisation but enhanced fMLP-induced O₂⁻ generation. Thus while adhesion molecules may play an as yet undefined role in priming transmigrating neutrophils, chemoattractant-induced actin polymerisation does not appear to be an essential component of this process.

1.8.7 Protein Phosphorylation and Neutrophil Priming

Protein phosphorylation is an important regulatory mechanism utilised by cytokines in signal transduction. Although most protein phosphorylation occurs on serine and threonine residues, tyrosine phosphorylation also plays an important role in the control of cellular function. The cytosolic oxidase component p47^{phox} becomes extensively phosphorylated on oxidase activation, the phosphorylation targets consisting of a group of serines in the carboxy-terminus of the peptide (El Benna et al., 1994); the phosphorylation of S379 in particular has been shown to be critical to oxidase activation (Faust et al., 1995). Although the kinase(s) responsible for this crucial phosphorylation event have not been identified, one possible candidate is the serine kinase mitogen activated protein kinase (MAPK), which must itself be activated by phosphorylation on both serine and tyrosine residues (Anderson et al., 1990).

Activation of neutrophils by fMLP and other stimuli is associated with increased tyrosine phosphorylation of multiple substrates (Rollet et al., 1994). Tyrosine phosphorylation has also been described in response to several priming agents, including GM-CSF (McColl et al., 1991), TNF α (Akimaru et al., 1992, Berkow and Dodson, 1988, Lloyds et al., 1995), Substance P (Lloyds et al., 1995) and PAF (Gomez-Cambronero et al., 1991). In several of these studies, the time-course of tyrosine phosphorylation was consistent with a

Figure 1.8

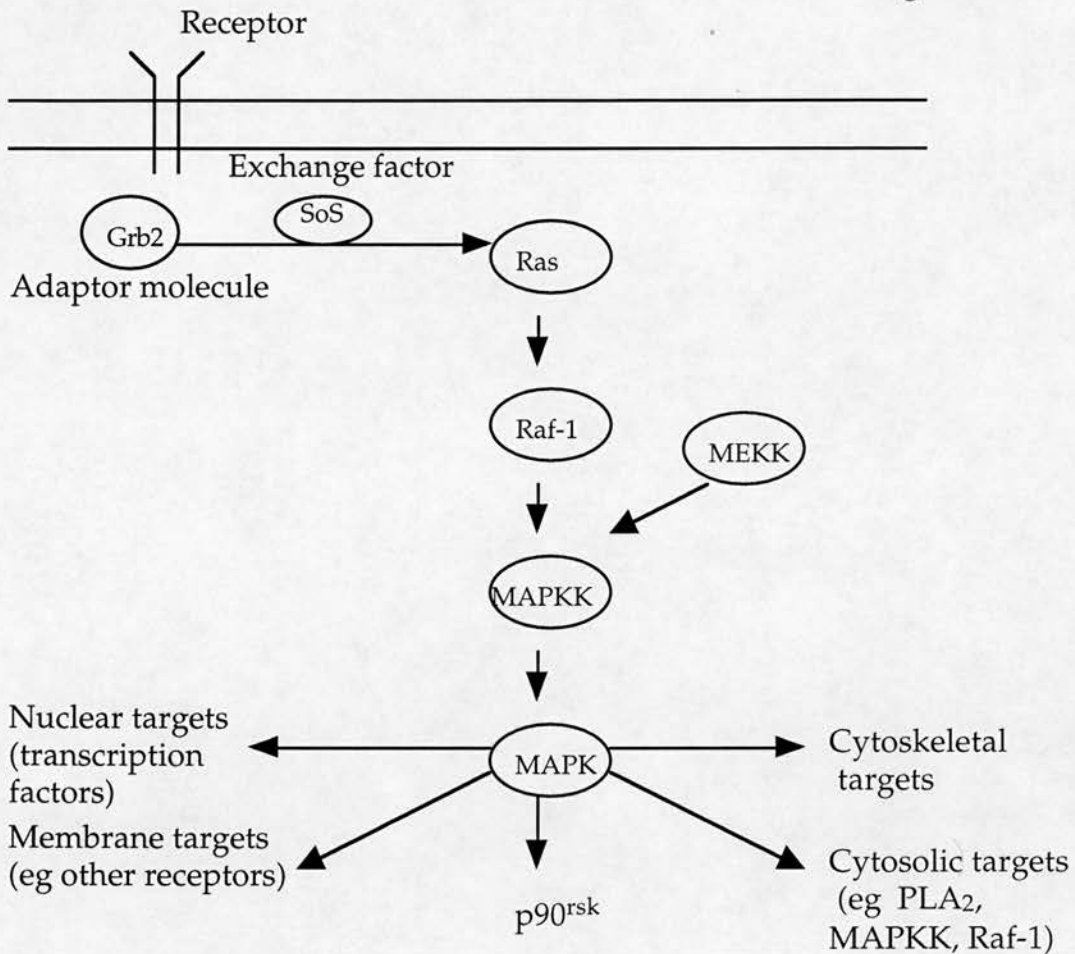


Figure 1.8 MAPK Signalling Cascade

Ligand-receptor interaction leads to activation of the small GTP-binding protein Ras via the adapter molecule Grb2 and the guanine nucleotide exchange factor SoS. Ras activates the serine/threonine kinase Raf-1, which can phosphorylate MAPK kinase (MAPKK); the latter phosphorylation can also be carried out by MEKK and possibly other kinases. Phosphorylation of both tyrosine and threonine residues of MAPK by MAPKK is required for full MAPK activation. Targets of MAPK include p90^{rsk}, a serine/threonine kinase whose substrates include transcription factors and ribosomal S6 protein, PLA₂, receptors, nuclear and cytoskeletal targets, and MAPKK and Raf-1 (negative feedback).

role in priming (although the study with GM-CSF demonstrated that tyrosine phosphorylation was detectable at 1-5 min and maximal at 15-30 min, clearly earlier than the establishment of maximal priming). Furthermore, manipulation of tyrosine phosphorylation levels within neutrophils by inhibition of tyrosine phosphatases (e.g. by orthovanadate) or tyrosine kinases (e.g. by genistein) resulted in priming and inhibition of priming respectively (Lloyds and Hallett, 1994).

Phosphorylation of MAPK has been reported secondary to priming concentrations of LPS (Fouda et al., 1995), GM-CSF (Okuda et al., 1992, Raines et al., 1994, Gomez-Cambronero et al., 1992) and PAF (Gomez-Cambronero et al., 1991). However, a study by Waterman and Sha'afi (1995) demonstrated that, although $\text{TNF}\alpha$ induced tyrosine phosphorylation of a 40 kDa protein that was initially thought to be MAPK, this phosphoprotein could not be identified using sera against known members of the MAPK family. Thus not all phosphoproteins with appropriate molecular mass can be assumed to be MAPK, and positive identification (e.g. by immunoprecipitation) as in all the above studies, is required to clarify the situation.

1.8.8 $\text{PtdIns}(3,4,5)\text{P}_3$ in Neutrophil Priming

In human neutrophils fMLP stimulation causes a rapid accumulation of the novel phospholipid $\text{PtdIns}(3,4,5)\text{P}_3$ (Traynor-Kaplan et al., 1988 and 1989) due to the action of the enzyme PI3K on the membrane phospholipid $\text{PtdIns}(4,5)\text{P}_2$ (Stephens et al., 1991). Initial reports demonstrated activation of PI3K by membrane-bound receptor tyrosine kinases resulting from recruitment of p110-p85 heterodimers to signalling complexes (Auger et al., 1989); this process was shown to be mediated by the SH_2 domains of the regulatory p85 subunit that bind specific phosphotyrosine residues on the

activated receptors (Rordorf-Nikolic et al., 1995). A separate PI3K isotype p110 γ , which does not associate with p85 but which is activated by both α and $\beta\gamma$ G-protein subunits, has also been cloned from human myeloid cells (Stoyanov et al., 1995).

fMLP-induced PtdIns(3,4,5)P₃ accumulation in human neutrophils was shown to be rapid (peak at approximately 10 sec, Stephens et al., 1993b), inhibited by pertussis toxin but not by genistein (Corey et al., 1993) and associated with little (Stephens et al., 1993b) or no (Vlahos and Matter, 1992) increase in PI3K activity detected in anti-phosphotyrosine antibody immunoprecipitates. The fungal metabolite wortmannin inhibited both forms of PI3K (Arcaro and Wymann, 1993, Okada et al., 1994a and 1994b, Stoyanov et al., 1995) and abolished the respiratory burst in response to fMLP without directly affecting NADPH oxidase activity (Baggiolini et al., 1987, Dewald et al., 1988). This evidence implicates PI3K, probably the recently described p110 γ isoform, in the signal transduction process linking receptor stimulation with oxidase activity.

The role of PI3K in priming is unknown. The priming agent GM-CSF was shown to stimulate the accumulation of PtdIns(3,4,5)P₃ in neutrophils; this response was maximal at 3 min, and was not delineated beyond 5 min (Corey et al., 1993), whereas priming with GM-CSF requires a more prolonged incubation, as discussed above. The GM-CSF response was inhibited by genistein but not by pertussis toxin, and was associated with the formation of protein tyrosine kinase-coordinated signalling complexes. In the same study, a second priming agent, TNF α , was reported not to affect PtdIns(3,4,5)P₃ accumulation in neutrophils. PAF and ATP, both of which rapidly prime neutrophils, have also been shown to lead to rapid accumulation of PtdIns(3,4,5)P₃ (Stephens et al., 1993a and 1993b). However,

Figure 1.9

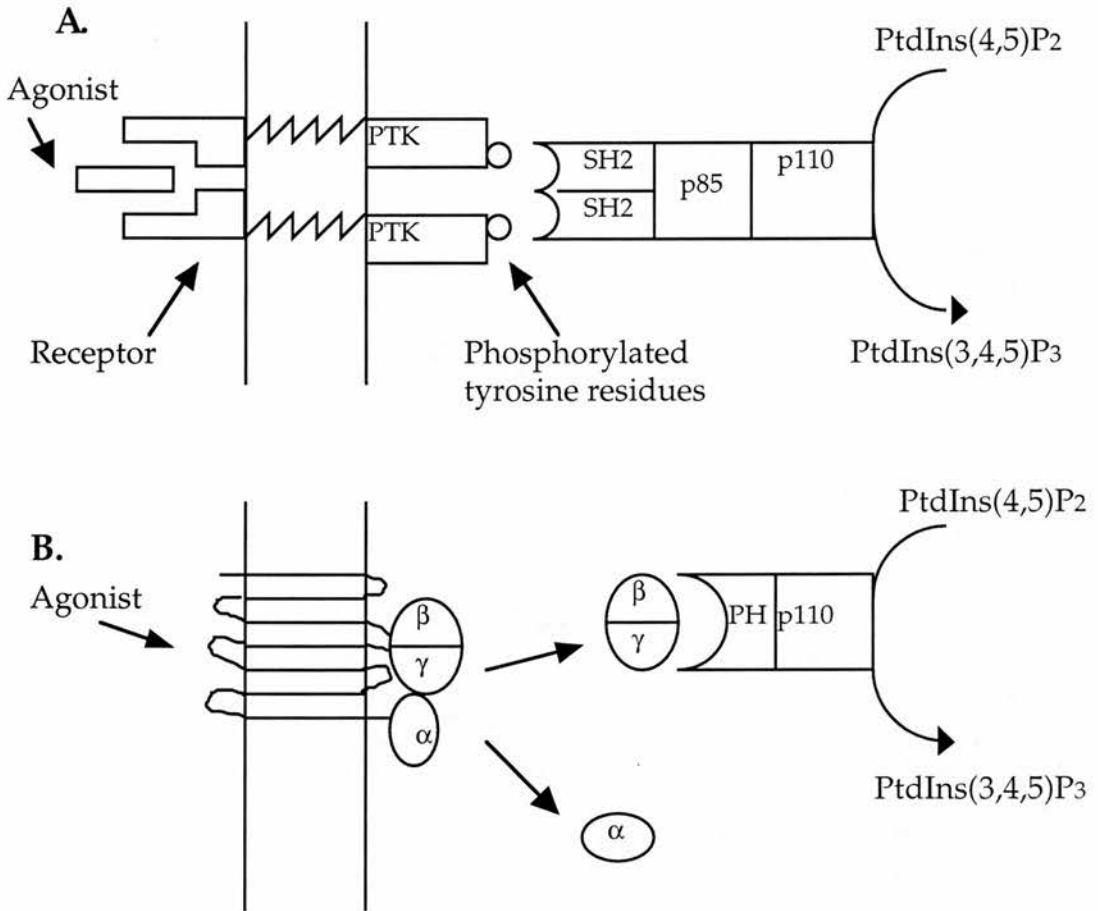


Figure 1.9 Signalling via PI3K

A). Receptors with intrinsic tyrosine kinase activity undergo autophosphorylation, and the phosphorylated tyrosine residues permit interaction with SH2 domains of the p85/p110 isoform of PI3K (receptors lacking tyrosine kinase activity may associate with src-type non-receptor kinases). The activated enzyme catalyses the conversion of PtdIns(4,5)P₂ to PtdIns(3,4,5)P₃.

B). Association of ligand with G-protein-linked receptor results in G-protein subunit dissociation; the p110γ PI₃K isoform possesses a pleckstrin homology (PH) domain, allowing interaction with, and activation by, the G-protein subunits.

no studies detailing the effect of priming agents on fMLP-stimulated PtdIns(3,4,5)P₃ accumulation have been published. The downstream targets of PtdIns(3,4,5)P₃ are uncertain, but may include the small GTP-binding proteins ras (Hu et al., 1995) and p21^{rac} (Hawkins et al., 1995), and PKC (Singh et al., 1993, Nakanishi et al., 1993, Toker et al., 1994). In addition to its role in signalling the respiratory burst, PI3K has been implicated in the control of actin polymerisation (Eberle et al., 1990) and early endosome fusion (Jones and Clague, 1995). Considerable research is ongoing in this rapidly expanding field.

1.8.9 Role of the Sphingomyelin Pathway in Neutrophil Priming

Recent evidence suggests that TNF α may employ the sphingomyelin pathway to effect a variety of its biological effects (reviewed in Kolesnick and Golde, 1994). Hydrolysis of plasma membrane sphingomyelin by sphingomyelinase (activated by interaction of TNF α trimers with p55 receptors, inducing receptor clustering) yields ceramide. Ceramide acts as a second messenger, stimulating a serine/threonine ceramide-activated protein kinase to transduce the cytokine signal, in part via the MAPK and stress-activated protein kinase (SAPK) cascades. Both ceramide and exogenous sphingomyelinase mimicked TNF α action and induced apoptosis in HL-60 cells and in the monocytic cell line U937 (Obeid et al., 1993, Jarvis et al., 1994). However, despite a report that sphingosine mobilises intracellular calcium in human neutrophils (Wong and Kwan-Yeung, 1993), Yanaga and Watson (1994) demonstrated that neither sphingomyelinase nor cell-permeable ceramide analogues stimulated or primed the neutrophil respiratory burst; furthermore, in the latter study, TNF α did not elevate the levels of ceramide-1-phosphate (produced from ceramide by the action of ceramide kinase). Thus it seems that in mature neutrophils, the

sphingomyelin pathway has a rather limited role, if any, in signal transduction, and is not a mediator of TNF α -induced priming. No reports have implicated this pathway in signalling by other priming agents.

1.8.10 Role of Protein Synthesis in Neutrophil Priming

A study by Hughes et al. (1987) demonstrated that the low rates of protein biosynthesis detectable in resting neutrophils could be augmented fivefold by treatment with 100 nM fMLP, while this agonist concentration induced only 8% of maximal (by 1 μ M fMLP) O₂⁻ production. These authors speculated that protein synthesis could have a role in priming (although most reports suggest that 100 nM fMLP induces maximal or near-maximal O₂⁻ production). Tennenberg et al. (1993) demonstrated that the protein synthesis inhibitor cycloheximide prevented priming by interferon- γ , and studies by Newberger et al. (1988) and Cassatella et al. (1990) revealed that incubation of neutrophils with interferon- γ (for 3 hrs in the latter study) led to increased synthesis of the oxidase component p91^{phox}. LPS was also shown to elevate p91^{phox} levels (Cassatella et al., 1990), but cycloheximide had no effect on neutrophil priming by either LPS (Guthrie et al., 1984) or by GM-CSF (DiPersio et al., 1988). The rapidity of priming secondary to agonists such as PAF, ATP and TNF α renders a role for protein synthesis extremely unlikely. Thus protein synthesis is not likely to be important in neutrophil priming except in the case of interferon- γ , and the role of this cytokine as a priming agent has been challenged by Pabst (1994) who suggested that contaminating LPS was responsible for its reported priming activity.

1.9 Aims

The principal aim of the work presented in this thesis was to investigate the signalling mechanisms involved in human neutrophil priming. During the course of this investigation, the following approaches were pursued.

1. Initial experiments were undertaken to determine the conditions for optimal priming of the respiratory burst using the inflammatory mediators LPS, TNF α and PAF as priming agents.
2. Since neutrophil adhesion and priming are closely linked (Nathan et al., 1989a, Waddell et al., 1994, Liles et al., 1995) the effects of these priming agents on the expression and function of cell surface adhesion molecules (particularly CD11b and CD62-L) were examined.
3. A detailed study of the effects of priming on the magnitude and kinetics of membrane $G_{i\alpha 2}$ upregulation was then performed ($G_{i\alpha 2}$ is the most abundant neutrophil G-protein (Goldsmith et al., 1987) and is functionally coupled to the fMLP-receptor (Gierschik et al., 1989)).
4. Finally, the potential role of inositol phospholipids in neutrophil priming was investigated. Both Ins(1,4,5)P₃ and PtdIns(3,4,5)P₃ seem to be essential for the neutrophil respiratory burst (Dewald et al., 1988), hence an extensive series of experiments was designed to study these two mediators in the context of neutrophil priming.

Chapter 2: Materials and Methods

2.1 Materials

The following reagents were all obtained from the Sigma Chemical Company (Poole, Dorset UK). fMLP was dissolved in dimethyl sulfoxide/phosphate-buffered saline (DMSO/PBS; 5 mg fMLP in 70 μ l DMSO made up to 11.35 ml with PBS) and stored at 1 mM at -20°C. LPS (from *E. Coli* serotype OIII:B4, γ -irradiated) was dissolved in PBS at 1 mg/ml, sonicated (ultrawave sonic bath, Belmont Instruments, Glasgow, Scotland) for 10 min and stored at -20°C; individual aliquots were further sonicated on thawing, immediately prior to use. Cytochrome C (prepared from horse heart) was stored at -20°C and dissolved in PBS pre-warmed to 37°C immediately prior to use. Superoxide dismutase (SOD) was dissolved in PBS at 7500 U/ml and stored at -20°C. PAF was dissolved in analar ethanol at 10 mM and stored at -80°C. Dextran-500 (molecular weight 500,000) was dissolved in sterile 0.9% saline (6% w/v) and stored at 4°C. Inositol hexakisphosphate (InsP₆, phytic acid, di-potassium salt) was dissolved immediately prior to use at 1 mM in PBS with 25 mM HEPES (N-2-hydroxyethylpiperazine-N'-ethane sulphonic acid, required to maintain pH 7.0-7.4). Dimethoxybenzidine (o-dianisidine HCl) dissolved at 1.25 mg/ml in distilled water was aliquoted and stored at -20°C. Adenosine triphosphate (ATP), sphingomyelinase (from *Bacillus cereus*, 50 U/ μ l in 50% glycerol/PBS, 50 mM Tris-HCl, pH 7.5), human albumin solution (10%), phorbol myristate acetate (PMA, dissolved at 1 mg/ml in DMSO and stored at -20°C), fura-2-tetraacetoxymethylester (fura 2-AM, dissolved in DMSO at 1 mM and stored at -20°C) and Nonidet P40 were also supplied by Sigma, as

was sterile, endotoxin-free PBS, pH 7.4, and PBS with 1.2 mM Ca^{2+} and 0.8 mM Mg^{2+} , pH 7.4 (PBS with divalent cations).

Human recombinant $\text{TNF}\alpha$ diluted in 1 ml sterile PBS was purchased from Genzyme (Cambridge, MA), stored at -80°C , and diluted further in PBS in accordance with the individual batch activity immediately prior to use. Percoll was obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). C_6 -ceramide (a cell-permeable ceramide analogue) from Matreya, Pleasant Gap, PA, was stored at 100 μM in ethanol at -20°C . Inositol 1,4,5-trisphosphate ($\text{Ins}(1,4,5)\text{P}_3$) was acquired from Research Biochemicals International (St Albans, Hertfordshire, UK), diluted in distilled water to 10 mM and stored at -80°C . Creatinine phosphokinase (Boehringer Mannheim, Lewes, East Sussex, UK) was dissolved in water at 2.5 units/ml and stored in aliquots at -80°C . Sterile 3.8% sodium citrate was from Phoenix Pharmaceuticals, Gloucestershire, UK. One-micron Fluoresbrite fluorescent latex microspheres were supplied by Polysciences (Warrington, PA).

Mouse monoclonal antibodies (mAb) to CD11b (44), CD11c (3.9), and CD35 (E11) were obtained from Serotec (Oxford, UK); anti-CD62-L (Leu-8) was from Becton Dickinson (Oxford, UK). Mouse mAb anti-CD11a (WAC 70) was generously donated by Dr J. Ross (Department of Surgery, Edinburgh University); fluorescein isothiocyanate (FITC)-conjugated F(ab')_2 fragments of rabbit anti-mouse immunoglobulin were from Dako (Buckinghamshire, UK). Rabbit antibody to $\text{G}_{i\alpha 2}$ (SPG, directed against the C-terminal decapeptide of $\text{G}_{i\alpha 2}$) was the generous gift of Professor Graham Milligan (Department of Biochemistry, University of Glasgow, Scotland); horseradish peroxidase-conjugated donkey anti-rabbit antibody was from the Scottish Antibody Production Unit (Dundee, Scotland). All antibodies were titrated to determine optimal binding concentrations.

[^{32}P] P_i (40 mCi/ml in HCl- and carrier-free aqueous solution), [^3H]Ins(1,4,5) P_3 (17-20 Ci/mmol), and [$\gamma\text{-}^{32}\text{P}$]GTP (2 $\mu\text{Ci}/\mu\text{l}$) were supplied by DuPont New England Nuclear (Stevenage, UK). Partisphere SAX HPLC columns were from Whatman Chromatography (Maidstone, UK) and polyethyleneimine (PEI) TLC plates were from CamLab (Cambridge, UK). Sterile phosphate-free HEPES-buffered Hanks solution (100 mM NaCl, 10 mM KCl, 1 mM MgCl_2 , 10 mM glucose, 1.5 mM CaCl_2 , 30 mM HEPES, pH 7.4) was prepared and supplied by GIBCO (Life Technologies, Renfrewshire, Scotland). All other chemicals were of molecular, reagent or cell culture grade and were obtained from BDH (Leicestershire, UK).

2.2 Isolation of Human Neutrophils by Dextran Sedimentation and Plasma/Percoll Density Gradient Centrifugation

Human neutrophils were purified according to the method of Haslett et al. (1985), utilising sterile, LPS-free (Chromagenix *Limulus* amoebocyte lysis assay kit) reagents and plastic ware (Falcon, Oxford, UK). Freshly drawn blood from healthy volunteers was anticoagulated (4 ml sterile 3.8% sodium citrate/36 ml blood) and centrifuged (300g, 20 min); the platelet-rich plasma supernatant was centrifuged (2500g, 20 min) to obtain platelet-poor plasma (PPP) or used to prepare autologous serum by the addition of CaCl_2 (final concentration 20 μM) at 37°C. Pelleted cells from the initial centrifugation were subjected to dextran sedimentation (5 ml 6% dextran-500 plus 25 ml 0.9% saline per tube, gently mixed and allowed to stand for 30-40 min). Leukocyte-rich plasma was aspirated from the sedimented red cells, centrifuged (500 g, 6 min), resuspended in 2 ml PPP and underlayered with 2 ml 42%, and 2 ml 51% plasma/Percoll (prepared from 90% Percoll in 0.9% saline, and PPP). Gradients were spun at 275g for 10 min and polymorphonuclear cells harvested from the 42%/51% Percoll interface.

Figure 2.1

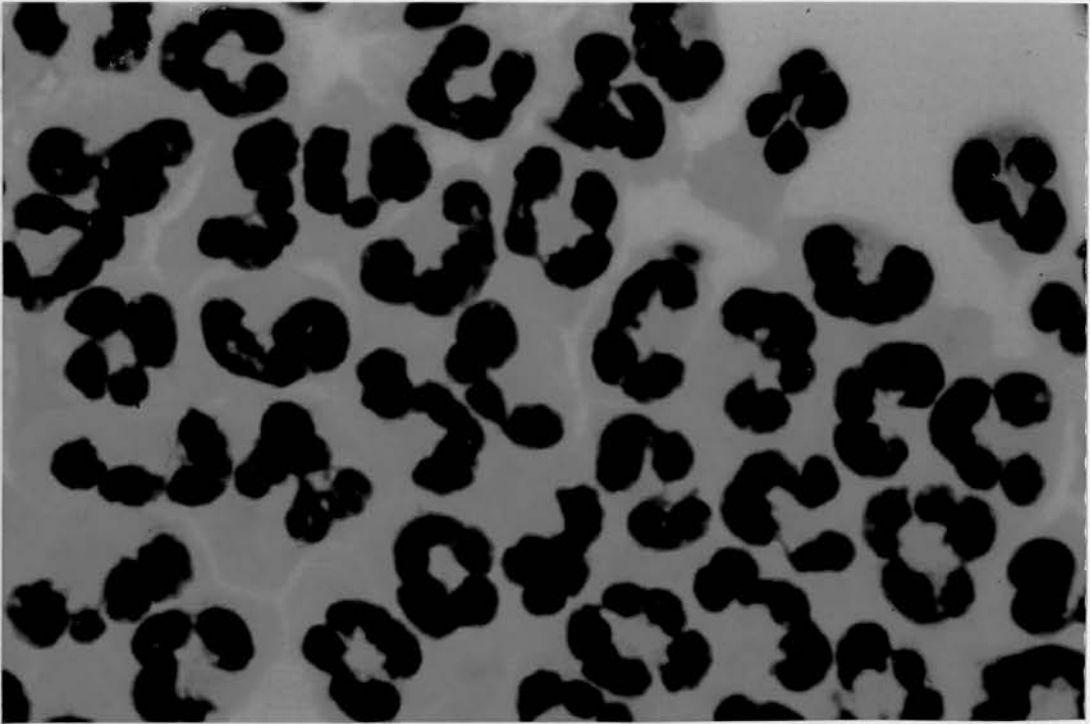


Figure 2.1 May-Grunwald-Giemsa-stained Cytospin Preparation of Human Neutrophils Isolated on Plasma/Percoll Gradients

Freshly drawn, anticoagulated human blood was subjected to sedimentation with 6% dextran prior to centrifugation through discontinuous 43%/51% plasma/Percoll gradients exactly as described in 2.2. Polymorphonuclear cells were harvested from the 42%/51% interface and washed sequentially in PPP, calcium-free PBS and PBS with calcium and magnesium. 100 μ l of the resulting cell suspension (approximately 10^7 /ml) was spun (300g, 3 min) onto a glass slide by means of a cytocentrifuge, and the resulting cytoprep was stained with May-Grunwald-Giemsa. Photograph courtesy of Miss Joanna Murray (Rayne Laboratory, University Medical School, Teviot Place, Edinburgh).

Mononuclear cells sedimented to the PPP/41% Percoll interface. Purified cells were washed sequentially in PPP, PBS, and PBS with calcium and magnesium; cell concentration was adjusted according to haemocytometer counts. All procedures were carried out at room temperature. Cells prepared by this method were routinely >99.9% viable (trypan blue exclusion), non-activated (minimal fMLP-stimulated superoxide anion (O_2^-) generation in the absence of a priming agent) and >95% pure as assessed by examination of May-Grunwald-Giemsa-stained cytocentrifuge preparations; mononuclear cell contamination was <0.1%. Since all polymorphonuclear leukocytes have very similar buoyant densities, density gradient centrifugation does not separate eosinophils or basophils from neutrophils; however, preparations generally contained <3% eosinophils, and if >5% eosinophils were present the cells were discarded. Basophils were seldom observed.

2.3 Measurement of Superoxide Anion Generation

Superoxide generation was quantified by the superoxide dismutase-inhibitable reduction of cytochrome C. Purified neutrophils isolated as detailed above were immediately resuspended at 11.1×10^6 /ml in PBS with calcium and magnesium, and 90 μ l aliquots were transferred to 2 ml LPS-free (Limulus assay) polypropylene Eppendorf tubes and placed in a Haake shaking water bath (100 cycles/min) pre-heated to 37°C. Thermal equilibrium was established (approximately 5 min) and priming agents or appropriate control buffers were added in a volume of 10 μ l for a designated time period. The incubation volume was subsequently adjusted to 1 ml by the addition of pre-warmed freshly prepared cytochrome C (final concentration 1.2 mg/ml in PBS with calcium and magnesium), fMLP (final concentration 100 nM) or PMA (final concentration 1 ng-1 μ g/ml) or vehicle

(final DMSO concentration $\leq 0.1\%$, v/v), with superoxide dismutase 375 U present in one of each set of quadruplicate samples. Shaking at 37°C was maintained for a further 10 min, at which time samples were placed on ice and centrifuged at 4°C, 10,000g for 5 min. The optical density of the supernatants was determined at 550 nm using a Pye-Unicam 8700 scanning spectrophotometer (with the baseline provided by 1.2 mg/ml cytochrome C), and the superoxide dismutase-inhibitable reduction of cytochrome C converted to nmols superoxide produced/ 10^6 cells using the extinction coefficient $21.0 \times 10^3 \text{M}^{-1}\text{cm}^{-1}$; the value obtained for the superoxide dismutase-containing samples was subtracted from the corresponding sample values to eliminate oxidation due to other respiratory burst products.

2.4 Assessment of Neutrophil Shape-Change

Neutrophil shape-change may be an indication of priming (Haslett et al., 1985). The percentage of shape-changed neutrophils within a population was estimated by means of the concomitant increase in forward scatter as measured by flow cytometry. Freshly isolated neutrophils (5×10^6 in 450 μl PBS with calcium and magnesium) were incubated at 37°C in 2 ml polypropylene Eppendorf tubes in a shaking water bath, with agonists (fMLP, $\text{TNF}\alpha$, LPS, PAF or appropriate vehicle controls) added in a volume of 50 μl . On completion of the incubation, cells were fixed by the addition of 0.5 ml 0.5% glutaraldehyde in PBS. Shape-change (5000 cells per sample) was determined using an EPICS Profile II (Coulter Electronics, Luton, UK) flow cytometer; shape-changed cells were defined as those with a forward scatter value greater than 90% of non shape-changed (rounded) cells. Fixed cells were also examined by light microscopy to verify the results obtained by flow cytometry; shape-change was defined as any deviation from spherical, and a minimum of 300 cells/sample were assessed.

Figure 2.2

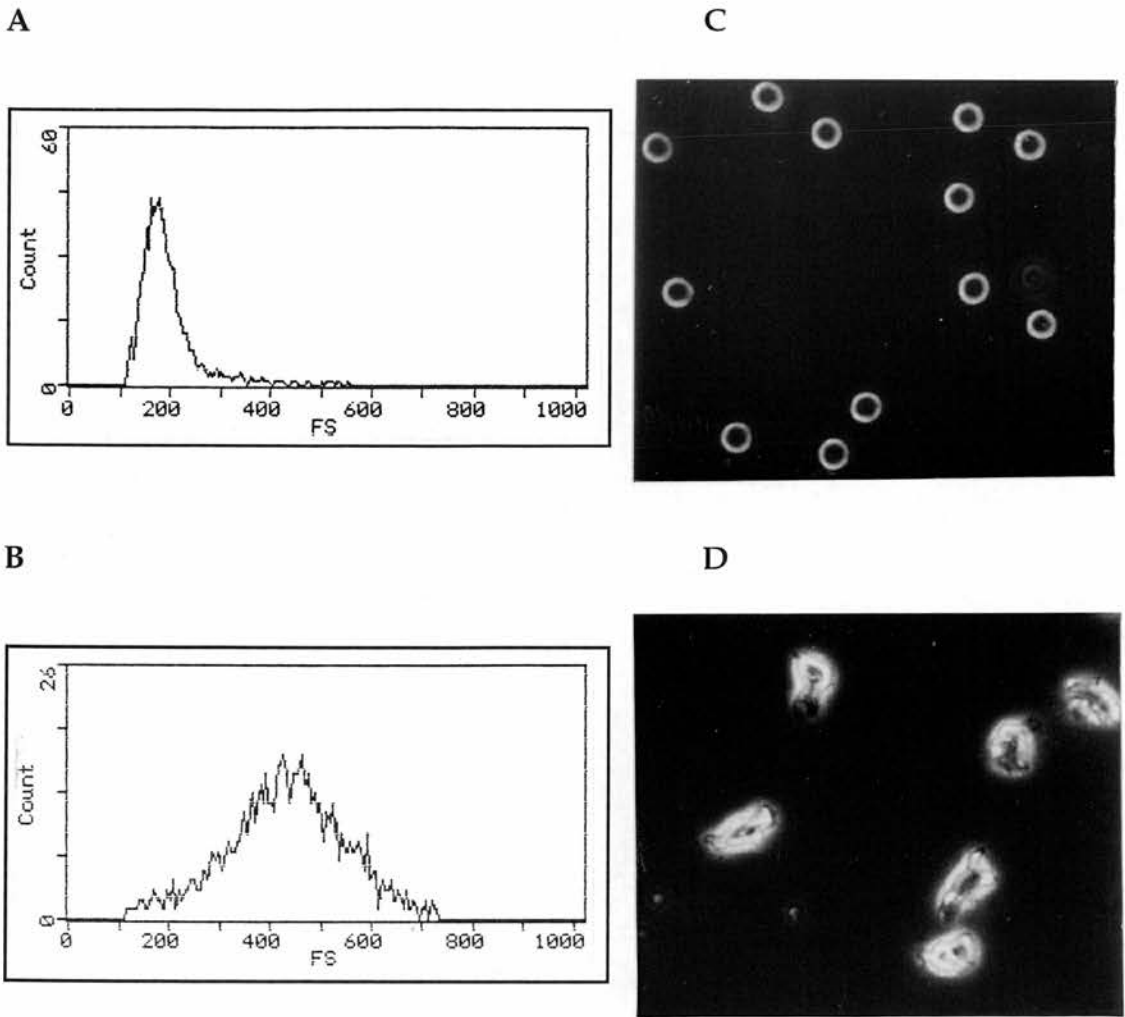


Figure 2.2 Assessment of Neutrophil Shape-Change by Flow Cytometry or Polarising Light Microscopy

Neutrophils isolated on plasma/Percoll gradients (2.2) were incubated at 37°C at a density of 5×10^6 /ml with fMLP 100 nM (B and D) or vehicle (A and C) for 15 min and fixed with 0.5% glutaraldehyde. A and B represent the forward scatter profiles of the fixed samples as assessed by flow cytometry; C and D illustrate the corresponding control (rounded) and fMLP-treated (shape-changed) cells seen by polarising light microscopy. Photographs courtesy of Dr Jiamin Qu (Department of Cellular Physiology, Babraham Institute, Cambridge).

2.5 Analysis of the Expression and Function of Neutrophil Cell Surface Adhesion Molecules

2.5.1 Determination of Neutrophil Adhesion Molecule Expression by Flow Cytometry

Expression of cell surface adhesion molecules was studied by indirect immunofluorescence, with primary monoclonal antibody (mAb) directed against the antigen of interest and FITC-conjugated second antibody bound to the primary antibody detected cytofluorimetrically. Purified human neutrophils were resuspended (10^7 cells/ml in PBS with divalent cations) and incubated at 37°C in a shaking water bath, with priming agents or appropriate vehicle controls added to give a total volume of 500 µl. After designated incubation times, sample volumes were adjusted to 1 ml by the addition of PBS or fMLP (100 nM final concentration). Following a further 10 min incubation at 37°C, the reactions were stopped by placing the cells on ice. Indirect immunofluorescence analysis was then performed as follows. Neutrophils (5×10^5 /well) were transferred to pre-chilled flexiwell plates (Becton Dickinson, Oxford, UK) and pelleted by centrifugation at 220g for 2 min. Cells were resuspended in 25 µl of saturating concentrations of primary mAb and incubated for 30 min at 4°C. Cells were then washed (x3) with PBS containing 0.2% bovine serum albumin (BSA) and 0.1% sodium azide, and incubated a further 30 min at 4°C with FITC-conjugated rabbit anti-mouse antibody (diluted 1 in 25 in PBS). Cells were again washed (x3) and then fixed in 1% formaldehyde. Samples were analysed using an EPICS Profile II (Coulter), and mean fluorescence values from a minimum of 2000 cells determined.

2.5.2 Determination of the Functional Capacity of Neutrophil CD11b by Binding of Albumin-Coated Latex Beads

The ability of neutrophils to bind albumin-coated latex beads was used as an index of CD11b function. Fluorescent latex microspheres (2.5% v/v stock solution) were washed three times in sterile PBS, resuspended and incubated at room temperature in human serum albumin (10 mg/ml) for ten minutes, and again washed three times in PBS. The resultant albumin-coated latex beads were resuspended at 0.75% (v/v) in PBS. 175 µl aliquots of freshly isolated neutrophils (10^7 /ml) were incubated in 2 ml Eppendorf tubes in a shaking water bath at 37°C, with agonists added in a volume of 25 µl to obtain the appropriate final agonist concentrations. 15 min prior to the termination of incubation (with 0.5 ml of 0.5% glutaraldehyde), 25 µl albumin-coated fluorescent latex beads were added to each reaction tube; for time-points of less than 15 min, the beads were added before the agonist. 30 min after the addition of glutaraldehyde, non-adherent beads were removed by three consecutive washes with PBS, and bead binding to PMN was assessed by cytofluorimetry (7500 cells analysed per sample).

2.6 Analysis of G-Protein Translocation and Activity in Control, Primed and Stimulated Human Neutrophils

2.6.1 Preparation of Neutrophil Membranes

A combination of nitrogen cavitation (cell disruption achieved by exposure to high pressure within a sealed chamber followed by rapid depressurisation) and high speed density gradient centrifugation was used to obtain neutrophil membranes; this method has previously been shown to yield ~ 75% plasmalemmal and 25% Golgi membranes with only minor contamination from endoplasmic reticulum, cytosol and granule membranes

(O'Flaherty et al., 1990, O'Flaherty and Rossi, 1993). Fresh human neutrophils were incubated (10^7 /ml) in the presence or absence of priming agents; reactions were stopped by pelleting the cells at 4°C followed by resuspension (2×10^7 /ml) in ice-cold relaxation buffer (100 mM KCl, 3 mM NaCl, 3.5 mM $MgCl_2$, 1 mM ATP, 10 mM PIPES, pH 7.3). The neutrophils were then subjected to nitrogen cavitation (400 p.s.i. nitrogen pressure, 4°C, 20 min) in a nitrogen bomb (Parr Instrument Company, Moline, USA), and the resulting cavitate was spun at 800g for 5 min at 4°C to remove undisrupted cells and debris. The supernatant was layered over 81% and 53% Percoll gradients prepared by mixing Percoll with dilution buffer - 1 M KCl, 30 mM NaCl, 35 mM $MgCl_2$, 10 mM ATP, 100 mM PIPES, 12.5 mM EGTA according to the following formula: lower (81%) gradient 13.2 ml Percoll, 1.5 ml dilution buffer/0.05 M HCl, 0.3 ml distilled water; upper (53%) gradient 5.1 ml Percoll, 1.5 ml dilution buffer/0.05 M NaOH, 8.4 ml distilled water; the gradients were centrifuged at 42,000g for 20 min (4°C) in a Beckman J2-MC centrifuge equipped with a JC-21 rotor. Plasma membranes were harvested from the upper interface layer, with granule membranes sedimenting to the lower interface. The plasma membrane fraction was resuspended in relaxation buffer and centrifuged for a further 60 min at 42,000g. The protein content of the washed membranes was estimated using the BCA protein assay kit (Pierce); protein yields ranged from 0.1 - 0.2 mg/ 10^8 neutrophils. Aliquots (0.2-0.4 mg protein/ml) were stored at -80°C prior to use.

2.6.2 Detection of G-Proteins in Membrane Fractions by Western Blotting

Membrane proteins were separated according to size by SDS PAGE, transferred to nitrocellulose, and G-proteins were visualised using a horseradish peroxidase-linked antibody detection system. The resolving gel

comprised 1.6 ml 50% glycerol in H₂O, 8 ml 30% acrylamide plus 0.8% bis-acrylamide, 6 ml buffer 1 (1.5 M Tris, 0.4% SDS, pH 8.8), 90 µl 10% ammonium persulphate (freshly prepared), 8.2 ml H₂O and 8 µl Temed; this solution was cast between clean gel plates and overlaid with 2 ml 0.1% SDS to exclude air, thereby promoting setting. When the resolving gel had set (about 2 hrs), the 0.1% SDS was poured off and the stacking gel (1.5 ml 30% acrylamide plus 0.8% bis-acrylamide, 3.75 ml buffer 2 (0.5 M Tris base, 0.4% SDS, pH 6.8) 150 µl 10% ammonium persulphate, 9.75 ml H₂O and 8 µl Temed) was layered over it. A gel comb was inserted into the stacking gel, which was allowed to set for about 2 hours, when the comb was removed. Neutrophil membrane proteins (5 µg or 10 µg) prepared and stored as described above were precipitated by the addition of 10 µl 2% Na deoxycholate, 250 µl trichloroacetic acid (TCA) and 750 µl H₂O, pelleted and resuspended in 15 µl 1 M Tris base plus 20 µl Laemmli loading buffer. The gel was placed in a gel tank with gel running buffer (72 g glycine, 15 g Tris base and 50 ml 10% SDS made up to 5 l with dH₂O) and the protein samples loaded with a 50 µl Hamilton syringe into the wells; markers of standard molecular weight were included on each gel. Electrophoresis (50 volts, 20 milliamps) was carried out overnight or until the dye front had run off the gel. The gel was carefully removed from the plates, discarding the stacking gel element, and placed in blotting buffer (72 g glycine, 15 g Tris base, 1 l methanol, the whole made up to 5 l with dH₂O). A nitrocellulose membrane was placed over the gel, and the gel plus membrane was sandwiched between 3 mm filter paper and foam. The gel sandwich was placed in a blotting tank, covered with blotting buffer, and the electrophoresed proteins were transferred to the nitrocellulose (100 volts, 20 milliamps for 90-120 min). Even sample loading was confirmed by staining the nitrocellulose blot with 24% Ponceau-S in 3% TCA; the red dye was removed by washing in

blotting buffer and subsequently dH₂O, and the nitrocellulose was placed in 5% gelatin in PBS for 2-3 hrs at 37°C to block the remaining areas of the membrane. After thorough washing with water the immunoblot was incubated overnight at 37°C with primary rabbit antibody (SPG, directed against the C-terminal decapeptide of G_{iα2}, supplied by Professor Graham Milligan) in 1% gelatin/0.2% Nonidet P40/PBS. The primary antibody was removed and the immunoblot washed (water, 0.2% NP40 in PBS, and PBS) prior to a further 3 hr incubation with horseradish peroxidase-conjugated donkey anti-rabbit IgG diluted 1 in 250 in 1% gelatin/0.2% Nonidet P40/PBS. The nitrocellulose was washed as above, and developed by the addition of 0.02% O-dianisidine in PBS (50 ml) plus 10 µl 40% hydrogen peroxide solution.

2.6.3 Assay of GTPase Activity in Membrane Fractions

GTPase activity was determined by the hydrolysis of [γ -³²P]GTP to free ³²Pi in the presence or absence of 100 µM GTP. The reaction mixture (final volume 100 µl) contained 0.05 µCi [γ -³²P]GTP, 10 mM creatinine phosphate, 10 U creatinine phosphokinase, 1 mM ATP, 0.1 mM App(NH)p, 1 mM ouabain, 100 mM NaCl, 5 mM MgCl₂, 2 mM dithiothreitol, 0.1 mM EDTA, 40 mM Tris base and 500 nM GTP, and the reaction was initiated by the addition of 4 µg membrane protein. For each sample, three sets of triplicate incubations were performed, with the addition of 100 nM fMLP to one set of triplicates and of 100 µM GTP to a further set. Reactions were terminated after 10 min at 37°C by the addition of ice-cold 5% activated charcoal in 10 mM phosphoric acid; the charcoal was pelleted (13,000g, 5 min) and the free ³²Pi present in the supernatant was determined by scintillation counting. High affinity GTP hydrolysis was calculated by subtraction of values

obtained in the presence of 100 μ M GTP, and results expressed as pmol free P_i generated per mg protein per minute.

2.7 Determination of Ins(1,4,5) P_3 Mass

Ins(1,4,5) P_3 mass was measured using the radioreceptor assay of Challiss et al. (1988) with minor modifications. The assay utilises an Ins(1,4,5) P_3 binding protein derived from bovine adrenal cortex (BAC), which is a rich source of a single, high affinity [3H]Ins(1,4,5) P_3 binding site; the displacement of [3H]Ins(1,4,5) P_3 by free Ins(1,4,5) P_3 from this site forms the basis of the assay.

2.7.1 Preparation of Ins(1,4,5) P_3 Binding Protein

Binding protein for use in the Ins(1,4,5) P_3 radioreceptor assay (see below) was prepared from decapsulated, demedullated bovine adrenal glands obtained from the local abattoir, as described by Challiss and co-workers (1988). The adrenal cortical tissue from 8-12 glands (60-80 g, kept at 4°C) was homogenised (Polytron tissue homogeniser, Polytron, Switzerland) in 8 volumes of homogenisation buffer (20 mM $NaHCO_3$, 1 mM dithiothreitol, pH 7.8) and centrifuged (Beckman J2-MC) at 5000g, 4°C for 10 min. The supernatant was aspirated and stored at 4°C while the pellet was re-homogenised in 4 volumes of buffer and the centrifugation step repeated. The 2 supernatant fractions were pooled and spun at 38,000g, 4°C for 20 min, and the pelleted protein resuspended in homogenisation buffer at 20 mg/ml (Pierce BCA protein assay kit, using bovine serum albumin as standard in accordance with the manufacturers' instructions) prior to aliquoting and storage at -20°C.

2.7.2 Preparation of Neutralised TCA Extracts from Human Neutrophils

Because of their negative charge and tendency to form protein associations, inositol polyphosphates partition poorly into the aqueous phase; in an acidic environment their negative charge and ability to form salt bridges is suppressed, enabling fuller partitioning and recovery. Hence a TCA extraction protocol was employed.

Neutrophils were suspended in PBS with divalent cations at a density of 32.2×10^6 /ml, and 225 μ l aliquots were placed in 2 ml polypropylene tubes in a shaking water bath at 37°C and allowed to reach thermal equilibrium. Priming agents (final concentrations: TNF α 200 U/ml, LPS 100 ng/ml or PAF 100 nM) or appropriate vehicle controls were added (25 μ l), and priming allowed to proceed for the appropriate time prior to stimulation with fMLP (final concentration 1-100 nM fMLP) or PBS (50 μ l). Reactions were stopped at 0-120 s by the addition of 60 μ l ice-cold 3 M TCA, and samples allowed to extract at 4°C for 20 min. The TCA extracts were vortex-mixed, spun at 13,000g for 5 min at 4°C, and 300 μ l supernatant combined with 75 μ l EDTA 10 mM, pH 7.0. Samples were removed from ice prior to the addition of 300 μ l freshly prepared 1,1,2-trichlorotrifluoroethane (freon)/tri-n-octylamine (1:1, v/v), vigorous vortexing and centrifugation at 15,000g for 5 min to allow phase partitioning. The pH of 200 μ l of upper (aqueous) phase was adjusted to 7.0 by the addition of 40 μ l 60 mM NaHCO₃, and the neutralised extracts stored at 4°C prior to analysis.

2.7.3. Ins(1,4,5)P₃ Mass Assay

Incubations were performed in 4 ml LP3 tubes at 4°C in a final volume of 120 μ l; all standards and samples were analysed in duplicate. 30 μ l sample, or 30 μ l distilled, deionised H₂O containing standard amounts of D-Ins(1,4,5)P₃ (0.3-300 nM final concentration in 120 μ l), or 1.2 μ moles (10 μ M) D-Ins(1,4,5)P₃

Figure 2.3

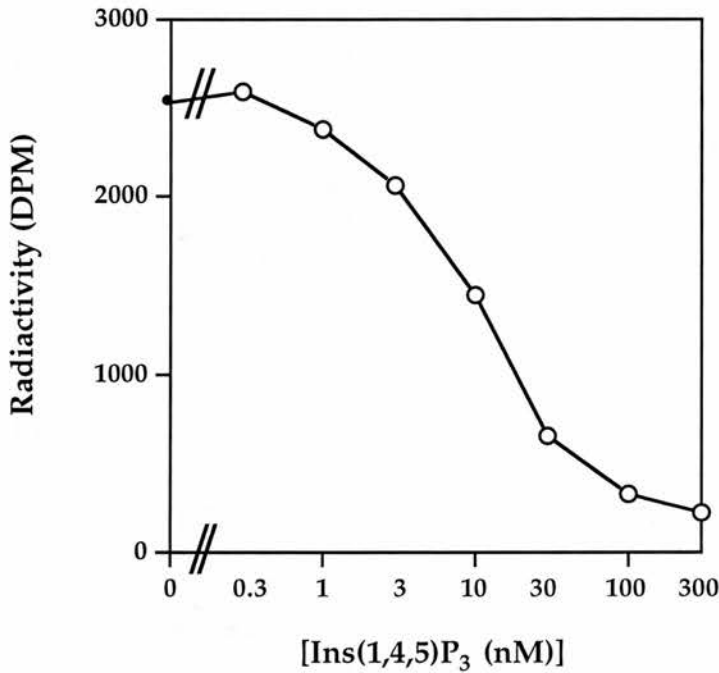


Figure 2.3 Inositol 1,4,5-trisphosphate Mass Assay: Standard Curve

Ins(1,4,5)P₃, 0-300 nM, was incubated at 4°C for 60 min with 0.6 mg Ins(1,4,5)P₃-binding protein and 8,000-10,000 d.p.m. [³H]Ins(1,4,5)P₃ as described in section 2.7.3. Bound and free [³H]Ins(1,4,5)P₃ were separated by filtration through Whatman GF/B filters; bound [³H]Ins(1,4,5)P₃ retained by the filters was quantified by liquid scintillation counting. Data represent the means of duplicate samples, and standard errors ($\leq 4.6\%$ of the mean) lie within the data points. ED₅₀=12.5 nM; non-specific binding=0.9%; [³H]Ins(1,4,5)P₃ bound in the absence of unlabelled Ins(1,4,5)P₃ (B₀)=2% of total radioactivity added.

to determine the non-specific binding, were combined with 30 μ l ice-cold buffer (100 mM Tris/HCl, 4 mM EDTA, pH 7.8) and 30 μ l [3 H]Ins(1,4,5)P₃ in dH₂O (8,000-10,000 d.p.m. per 30 μ l). The reactions were commenced by the addition of 30 μ l (0.6 mg protein) BAC Ins(1,4,5)P₃ binding protein with gentle vortexing, and allowed to reach equilibrium (60 min at 4°C). Separation of bound from free [3 H]Ins(1,4,5)P₃ was achieved by filtration through Whatman GF/B filters, washing with 4 x 3 ml of ice cold filtration buffer (25 mM Tris/HCl, 1 mM EDTA, 5 mM NaHCO₃, pH 7.8). Individual filter discs were placed in scintillation vials, covered with 4 ml Emulsafe scintillation fluid, and following a 12 hour extraction period the radioactivity associated with bound [3 H]Ins(1,4,5)P₃ was determined by liquid scintillation counting. Unknown Ins(1,4,5)P₃ concentrations were derived from the internally generated standard curve using a Packard radioimmunoassay programme (Securia II). Standard curves (see Figure 2.3) were highly reproducible, with an ED₅₀ of 13.4 \pm 0.9 nM and a non-specific binding of 0.85 \pm 0.08% (n=6).

2.8 Quantification of [32 P]Phosphatidylinositol 3,4,5-trisphosphate Accumulation in Human Neutrophils

Lipids from [32 P]P_i-labelled neutrophils primed with TNF α and stimulated with fMLP were extracted, deacylated and separated by TLC and/or anion exchange HPLC to allow quantification of [32 P]PtdIns(3,4,5)P₃.

2.8.1 Priming and Stimulation of [32 P]P_i-Labelled Neutrophils

Neutrophils take up [32 P]P_i poorly, and since little PtdIns(3,4,5)P₃ is present even in stimulated cells, a high specific activity was required for adequate labelling. Loading of cells with [32 P]P_i was optimised by performing the incubation in sterile phosphate-free HEPES-buffered Hanks solution (100

mM NaCl, 10 mM KCl, 1 mM MgCl₂, 10 mM glucose, 1.5 mM CaCl₂, 30 mM HEPES, pH 7.4). Freshly isolated neutrophils were washed twice in phosphate-free buffer, resuspended at 10⁸ cells in 2.25 ml and incubated with 5 mCi [³²P]P_i in 125 µl water, together with an equal volume (125 µl) of sterile 1.8% saline to maintain isotonicity. The cells were incubated at 37°C in a shaking water bath for 70 min, washed (2 x 10 ml phosphate-free buffer) and resuspended in 1.2 ml phosphate-free buffer. The cells were then aliquoted (100 µl) into 2 ml Eppendorf tubes, incubated for 30 min with TNFα (40 µl, final concentration 200 U/ml) or buffer and stimulated for 10 s or 60 s with fMLP (40 µl, final concentration 100 nM). Reactions were stopped by the addition of 675 µl methanol/chloroform (2:1, v/v).

2.8.2 Measurement of fMLP-stimulated PtdIns(3,4,5)P₃ in TNFα-primed and Unprimed Human Neutrophils

Samples prepared as detailed above were acidified by the addition of 158 µl 2.4 M HCl containing 5 mM tetrabutylammonium sulphate and partitioned using 675 µl chloroform. The lower phase was removed, washed with 658 µl synthetic upper phase (methanol:1 M HCl:chloroform, 48:47:3, v/v/v), and dried under vacuum. The lipids were deacylated to render them water-soluble by the addition of 200 µl of monomethylamine reagent (prepared by bubbling monomethylamine gas into a 4:3:1 (v/v/v) mixture of methanol: water: n-butanol in a marked vessel placed in dry-ice bath (monomethylamine gas is explosive at room temperature) to increase the liquid volume 1.625-fold; aliquots of the reagent were stored at -80°C in tightly sealed tubes and removed immediately prior to use) to each sample. Following vigorous vortexing, the samples were placed in a 53°C water bath for 30 min, with further vortexing at 15 min. Samples were again dried under vacuum, and the deacylated lipids were extracted in the presence of

200 μ l phytate hydrolysate (12 μ g phosphorus per sample) with 240 μ l n-butanol/light petroleum/ethylformate (20:4:1, v/v/v). The lower phase was removed and dried under vacuum and the [32 P]glycerophosphoesters were redissolved in 2 μ l 20 mM HCl, 1 mM K_2PO_4 and chromatographed on polyethyleneimine-cellulose plates [Stephens et al., 1993]: the plates were developed in 0.48 M HCl, and the radioactivity in GroPInsP₃ and GroPInsP₂ spots quantified by means of a phosphoimager (Molecular Dynamics). To separate isomers of [32 P]glycerophosphoesters (i.e. GroPIns3P from GroPIns4P or GroPIns(4,5)P₂ from GroPIns(3,4)P₂), the [32 P]glycerophosphoesters were redissolved in 2 ml H₂O and resolved by anion-exchange chromatography; a Partisphere 5-SAX column was eluted at 1 ml/min with a gradient of 2.5 M NaH₂PO₄ (pH 3.8) with NaOH 0-100%, samples being collected every 30 s

To confirm that the above [32 P]-labelling protocol did not induce neutrophil priming, cells from the same preparation were incubated under identical conditions in parallel with the above samples and assayed for O₂⁻ release rather than [32 P]PtdIns(3,4,5)P₃. After confirming that the [32 P]P_i labelling protocol did not induce priming or affect the subsequent degree of activation, the [32 P]P_i component was replaced by sterile H₂O in subsequent parallel assays to assess O₂⁻ generation.

2.8.3 Effects of TNF α and fMLP on Neutrophil PI3K Activity in Anti-phosphotyrosine Immunoprecipitates.

Human neutrophils (3x10⁷/ml in PBS with divalent cations) were incubated in the presence of TNF α 200 U/ml or vehicle for 30 min and stimulated with fMLP 100 nM or vehicle for 60 s. The reactions were terminated by centrifugation at 4°C (4 s, 15,000g) and snap-freezing in liquid nitrogen. Immunoprecipitation and measurement of PI3K activity were carried out by

Dr L. Stephens (Department of Cell Signalling, Babraham Institute, Cambridge).

Samples were resuspended in 1 ml ice-cold lysis buffer (137 mM NaCl, 2.7 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂, 1% (w/v) NP40, 10% (w/v) glycerol, 1 mg/ml BSA, 20 mM Tris, 0.5 mM orthovanadate, and protease inhibitors (0.2 mM PMSF 10 µg/ml leupeptin, pepstatin and aprotinin, Sigma) pH 8.0), vortexed and placed on ice for 10 min.. Following centrifugation (30 min, 4500 g, 0°C), 0.8 ml aliquots were mixed with 8 µl antiphosphotyrosine monoclonal antibody (PY20, ICN) and 40 µl of a 1/1 suspension of protein A-sepharose CL4B beads (Pharmacia) pre-equilibrated for 2 hr in lysis buffer. After 2 hr at 0°C the beads were washed at 4°C as follows: three times with lysis buffer, twice with 0.5 mM LiCl, 0.1 mM Tris, pH 8.0, once with 0.15 mM NaCl, 10 mM Tris, 1 mM EDTA, pH 7.6 and once with 20 mM HEPES, 1 mM DTT, 5 mM MgCl₂, pH 7.6. The supernatants were discarded and 40 µl ice-cold assay buffer (20 mM β-glycerophosphate, 5 mM pyrophosphate, 30 mM NaCl, 1 mM DTT, pH 7.2) and 20 µl PtdIns/cholate (3 mg/ml PtdIns, 1% cholate in assay buffer) were added and the samples transferred to a 37°C water-bath. After 5 min, 40µl of a solution containing 3 µM ATP, 7.5 mM MgCl₂ and 0.25 mCi/ml [γ -³²P]ATP (Amersham) was added to initiate the assay. The reactions were quenched at 15 min by the addition of 500 µl chloroform/methanol/H₂O (97.88 ml chloroform/methanol, 1/2 (v/v) plus 2.12 ml H₂O) and the samples were mixed thoroughly. Finally, 489 µl chloroform and 114 µl 2.4 M HCl, 5 mM tetrabutylammonium sulphate were added, and the extraction procedure was completed exactly as described in (2.8.2) above. The extracted lipids were finally deacylated and resolved by HPLC (2.8.2).

Chapter 3: Priming of Human Neutrophil Superoxide Anion Generation

3.1 Introduction

Priming refers to the process whereby a greatly enhanced activation response is seen following the sequential addition of priming and activating agents. Activation of a primed neutrophil causes translocation of the cytosolic components of the NADPH oxidase enzyme system (p47^{phox}, p67^{phox} and the GTP-binding protein p21^{rac}) to the neutrophil membrane (to which the flavocytochrome b₅₅₈ is already localised) and the resultant generation of reactive oxygen species (the 'respiratory burst', Chapter 1.3), prominent amongst which is the superoxide anion (O₂⁻). Oxidase activity can be estimated by measuring several different parameters: 1) the increase in oxygen consumption using an oxygen electrode, (e.g. Berkow et al., 1987), 2) luminol- or lucigenin-enhanced chemiluminescence (e.g. Macphee et al., 1993), 3) fluorimetric measurement of hydrogen peroxide production (e.g. Nathan, 1987) and 4) measurement of superoxide production as a) the superoxide dismutase-inhibitable reduction of cytochrome C (e.g. Guthrie et al., 1984), as b) the production of formazan by reduction of nitroblue tetrazolium (e.g. Yanaga and Watson, 1994), or c) cytofluorimetrically as the reduction of dihydrorhodamine (e.g. Stocks et al., 1995). In general the preferred method is that of cytochrome C reduction, since it is both simple and quantitative (1 nmol O₂⁻ reduces 1 nmol cytochrome C, producing an increase in the absorbance at 550 nm); it measures extracellular O₂⁻ release, the fraction most likely to lead to tissue injury.

The importance of neutrophil O₂⁻ generation in the microbicidal process is emphasised by the hereditary condition chronic granulomatous disease;

mutations in the genes encoding individual components of the NADPH oxidase result in defective production of O_2^- , and patients suffer from recurrent life-threatening infections (Finn et al., 1990). Inappropriate or excessive O_2^- production may be involved in a number of disease processes: for example, ischaemia-reperfusion injury (Grisham et al., 1986), ARDS (e.g. Braude et al., 1986) and vasculitis (Keogan et al., 1991). Furthermore, O_2^- generation is the most 'primable' of the neutrophil's array of responses; production induced by the bacterial peptide fMLP, for example, is minimal in unprimed cells, but can be increased 10-20 fold by prior exposure of the neutrophil population to the more potent priming agents (e.g. Guthrie et al., 1984, Berkow et al., 1987, and see below). In contrast, fMLP-induced degranulation (Fittschen et al., 1988) and production of bioactive lipids (e.g. leukotriene B_4 , Doerfler et al., 1989) are enhanced to a much lesser extent by priming. Some pharmacological agents, such as the phorbol ester PMA (which activates PKC directly) cause near-maximal O_2^- generation alone, with only a slight increase on priming. The augmentation of the fMLP-induced O_2^- response is thus the 'gold-standard' for assessment of neutrophil priming.

Priming activity has been ascribed to a wide variety of substances; in this work I have focused on those with a probable physiological/pathological role. Bacterial lipopolysaccharide (LPS, endotoxin) was the first neutrophil priming agent to be recognised (Guthrie et al., 1984): $TNF\alpha$ and PAF are also well-established priming agents under certain conditions (Table 3.1). Inositol hexakisphosphate (phytic acid) has recently been suggested to act as a priming agent (Eggleton et al., 1991) - since large stores of this compound exist in mammalian cells (5-15 μM , Szevergold et al., 1987) and are released from dying or effete cells, this is a potentially important mechanism for modulation of local inflammatory processes. Finally, controversy exists

Table 3.1

Concentration- and Time-dependence for Priming of Human Neutrophil O_2^- Generation by a Series of Priming Agents

Agent	Concentration Range;Time	PMN	Reference
LPS	10 ng-1 μ g/ml; 30-120 min	Human	Guthrie et al., 1984
TNF α	10-500 U/ml; 5-60 min	Human	Berkow et al., 1987
GM-CSF	0.1-100 nM; 60-240 min	Human	Weisbart et al, 1986a
PAF	10 nM-1 μ M; 1-60 min	Human	Engleberger et al., 1987
Ionomycin	10 nM-1 μ M; 1-10 min	Human	Finkel et al., 1987

concerning the effects of PDGF on multiple neutrophil functions (see Section 3.8). The aims of the work presented in this chapter were to confirm or refute these substances as priming agents, and to establish optimal conditions for priming in terms of agonist-specific requirements for time to prime, and agonist concentration.

3.2 Priming of O_2^- Generation by LPS

3.2.1 LPS Priming of fMLP- and PMA-mediated O_2^- Generation

Incubation with the formylated bacterial tripeptide fMLP alone produced only minimal O_2^- release from human neutrophils, but its effects were augmented (primed) by preincubation with LPS under the conditions documented (Figures 3.1-3.3). In contrast, the phorbol ester PMA induced a massive release of O_2^- (greater than the primed fMLP response), that was not primed to any great extent by LPS (Figure 3.1) even when sub-maximal concentrations of PMA (≤ 10 ng/ml) were employed. fMLP, rather than PMA or related phorbol esters, was therefore used in the majority of subsequent experiments.

3.2.2 Serum-dependence of LPS Priming

The biological effects of LPS are profoundly affected by the serum factor lipopolysaccharide-binding protein (LBP) which combines with LPS to promote interaction with CD14 (Tobias et al., 1986), thought to be involved in transducing the signal to the cell interior. In the absence of this serum factor, responses to LPS are diminished both in terms of magnitude and rapidity of onset. To assess the serum-dependence of the LPS effect in our system, fMLP-stimulated O_2^- generation was determined in LPS-primed and unprimed cells in the presence or absence of 1% autologous serum (heat-inactivated at 56°C for 30 min to eliminate the effects of complement components). As can be seen from Figure 3.2, serum greatly augmented the priming effect of LPS at 1 hour (13.9-fold versus 7.2-fold augmentation of the response to fMLP alone); however, O_2^- generation by control (unprimed, unstimulated) cells and by cells treated with fMLP or with LPS alone was also enhanced (5.5, 6.2 and 2.9-fold increases respectively). In view of this

Figure 3.1

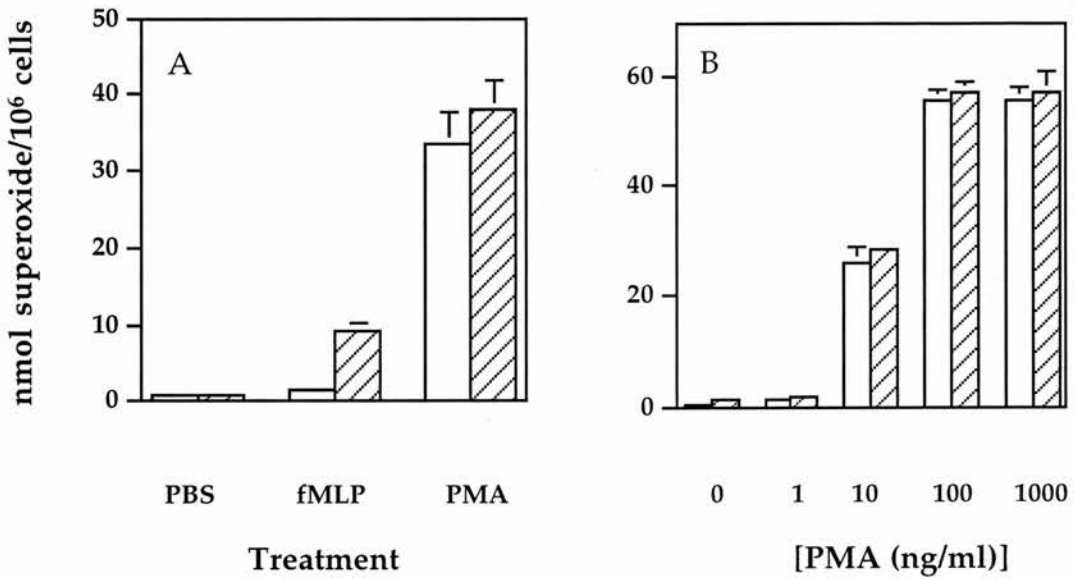


Figure 3.1 LPS Priming of fMLP- and PMA-stimulated O_2^- Generation in Human Neutrophils.

Human neutrophils (10^6 in 90 μ l PBS with calcium and magnesium) were incubated for 60 min in the presence (hatched bars) or absence (open bars) of LPS 100 ng/ml at 37°C in a shaking water bath. After 1 hr the incubation volume was adjusted to 1 ml by the addition of pre-warmed cytochrome C (final concentration 1.2 mg/ml) and agonists. Graph A documents the effects of LPS pre-treatment on fMLP- (100 nM, 10 min) and PMA- (10 ng/ml, 10 min) induced O_2^- generation, and graph B the effects of LPS on PMA- (1 ng-1 μ g/ml, 10 min) induced O_2^- generation, measured by the superoxide-dismutase-inhibitable reduction of cytochrome C as described in (2.3). Data represent the mean \pm sem of n=3 experiments, all performed in triplicate.

Figure 3.2

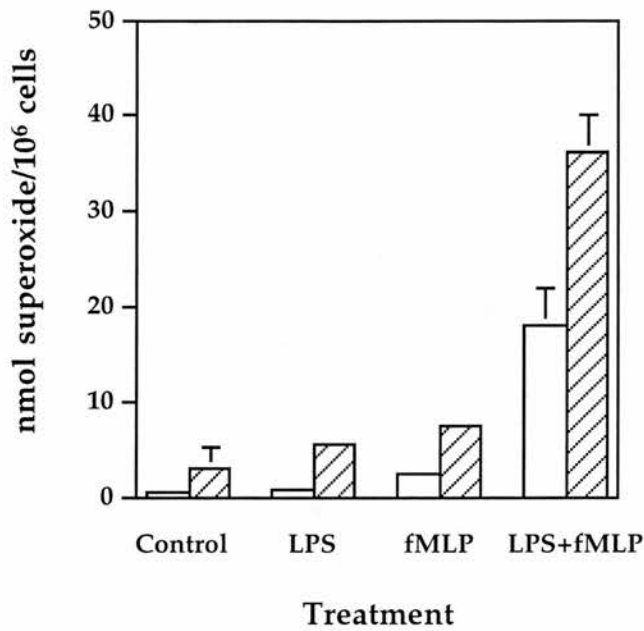


Figure 3.2 Effect of Serum on LPS Priming of fMLP-stimulated O_2^- Generation in Human Neutrophils

Human neutrophils (10^6 in 90 μ l PBS with calcium and magnesium) were incubated at 37°C in the presence (hatched bars) or absence (open bars) of 1% heat-inactivated autologous serum, with or without LPS 100 ng/ml for 1 hr prior to the addition of pre-warmed cytochrome C (final concentration 1.2 mg/ml) and of fMLP (final concentration 100 nM) or vehicle in a final volume of 1 ml. SOD (375 U) was included in one of each set of quadruplicate incubations, and O_2^- generation was quantified by the superoxide dismutase-inhibitable reduction of cytochrome C as described in (2.3). Data represent mean \pm sem of 3 separate experiments, each performed in triplicate.

priming/stimulatory effect of serum, the majority of further experiments were carried out in the absence of serum; experiments in which serum was included incorporated additional serum controls.

3.2.3 Concentration-dependence of LPS Priming

A concentration-response curve for LPS priming of fMLP-stimulated O_2^- generation was constructed utilising concentrations of LPS ranging from 1 ng/ml to 1 μ g/ml in the absence of serum. From Figure 3.3 it can be seen that increasing concentrations of LPS induced a near-maximal response at 100 ng/ml (EC_{50} 35 ± 14 ng/ml); elevating the concentration of LPS to 1 μ g/ml resulted in little increase in the degree of priming achieved, but some direct activation of O_2^- production in the absence of fMLP was observed. LPS 100 ng/ml was hence selected to prime neutrophils in the majority of future experiments.

3.3 Priming of O_2^- Generation by $TNF\alpha$ and by PAF

Both $TNF\alpha$ and PAF have been reported to prime neutrophils (Berkow et al., 1987; Engleberger et al., 1987). $TNF\alpha$ was found to be a potent and effective priming agent (Figure 3.4); preincubation with this cytokine for 30 min augmented fMLP-stimulated O_2^- generation at 20-20000 U/ml (EC_{50} $> 216 \pm 48$ U/ml); however the higher activities also induced O_2^- release in the absence of further stimulation. It should be noted that neutrophils were non-adherent, being kept in suspension by the use of a shaking water-bath and by the use of polypropylene surfaces; $TNF\alpha$ is a powerful activating agent for adherent neutrophils (Nathan, 1987). $TNF\alpha$ 200 U/ml was used in further experiments, since this activity induced priming with minimal direct activation. PAF was also found to be a powerful priming agent (EC_{50} $> 24 \pm 16$ nM); Figure 3.5 illustrates that concentrations as high as 1 μ M PAF led

Figure 3.3

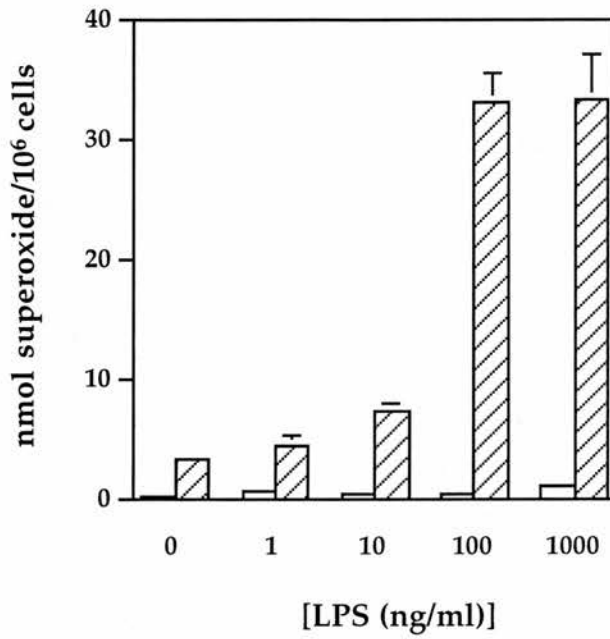


Figure 3.3 LPS Priming of fMLP-stimulated Superoxide Anion Generation in Human Neutrophils: Concentration-response

Human neutrophils (10^6 in 90 μ l PBS with calcium and magnesium) were incubated at 37°C in the presence (hatched bars) or absence (open bars) of LPS 0-1 μ g/ml, for 1 hr prior to the addition of cytochrome C (final concentration 1.2 mg/ml) and of fMLP (final concentration 100 nM) or vehicle in a final volume of 1 ml. SOD (375 U) was included in one of each set of quadruplicate incubations, and superoxide release was determined by the superoxide dismutase-inhibitable reduction of cytochrome C as described in (2.3). Data represent mean \pm sem of 3 separate experiments, each performed in triplicate.

Figure 3.4

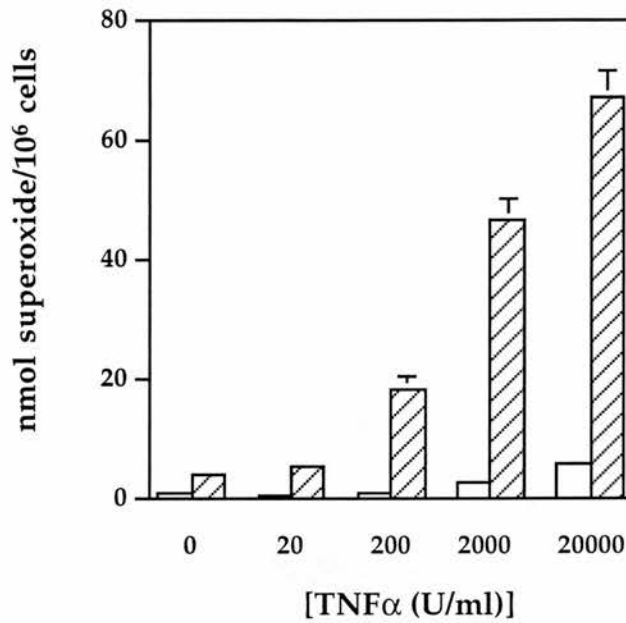


Figure 3.4 TNF α Priming of fMLP-stimulated O₂⁻ Generation in Human Neutrophils: Concentration-response

Human neutrophils (10⁶ in 90 μ l PBS with calcium and magnesium) were incubated at 37°C in the presence (hatched bars) or absence (open bars) of TNF α 0-20,000 U/ml for 30 min prior to the addition of pre-warmed cytochrome C (final concentration 1.2 mg/ml) and of fMLP (final concentration 100 nM) or vehicle in a final volume of 1 ml. SOD 375 U was included in one of each set of quadruplicate incubations, and superoxide release measured as described in (2.3). Data represent mean \pm sem of 3 separate experiments, each performed in triplicate.

Figure 3.5

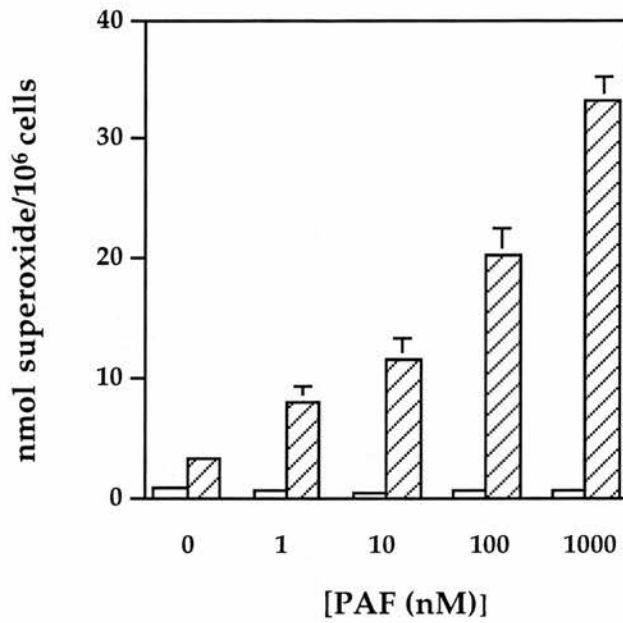


Figure 3.5 PAF Priming of fMLP-stimulated O_2^- Generation in Human Neutrophils: Concentration-response

Human neutrophils (10^6 in 90 μ l PBS with calcium and magnesium) were incubated at 37°C in the presence (hatched bars) or absence (open bars) of PAF 0-1 μ M, for 10 min prior to the addition of cytochrome C (final concentration 1.2 mg/ml) and of fMLP (final concentration 100 nM) or vehicle in a final volume of 1 ml. SOD (375 U) was included in one of each set of quadruplicate incubations. After 15 min cells were pelleted at 4°C and the optical density of the supernatants at 550 nm determined by scanning spectrophotometry; superoxide generation was calculated as described in (2.3). Data represent mean \pm sem of 3 separate experiments, each performed in triplicate.

to little observable stimulation of the respiratory burst, whilst concentrations as low as 1 nM were seen to prime. 100 nM PAF was used in subsequent experiments, since this concentration induced a degree of priming similar to that seen with LPS 100 ng/ml and with TNF α 200 U/ml.

3.4 Time-course for the Priming of Neutrophil O₂⁻ Generation by LPS, TNF α and PAF

To determine the optimal time-intervals required to establish the primed state for each agonist, freshly isolated neutrophils were incubated for 0-120 min with LPS 100 ng/ml (in the presence and absence of 1% autologous heat-inactivated serum), TNF α 200 U/ml or PAF 100 nM, prior to determination of the stimulated O₂⁻ response. These comparisons were all made using a single population of neutrophils. Figure 3.6 illustrates that the onset of priming was most rapid in the case of PAF, an effect being observed after a preincubation of only 1 min; the priming effect was maximal at 10-60 min and had declined somewhat by 120 min. Incubation with TNF α also primed neutrophils rapidly, priming being apparent by 10 min and maximal by 30-60 min. The inclusion of serum caused a profound alteration in the time-course of LPS-induced priming (Figure 3.6); in its absence, priming was not observed before 60 min had elapsed and was on-going at 120 min, but when both LPS and serum were present priming was seen at 30 min and was maximal by 60 min.

3.5 Lack of Effect of Recombinant Platelet-derived Growth Factor (PDGF) on Human Neutrophil O₂⁻ Generation

Conflicting reports exist concerning the effects of PDGF on multiple neutrophil functions (see 3.8, below); we therefore examined the ability of

Figure 3.6

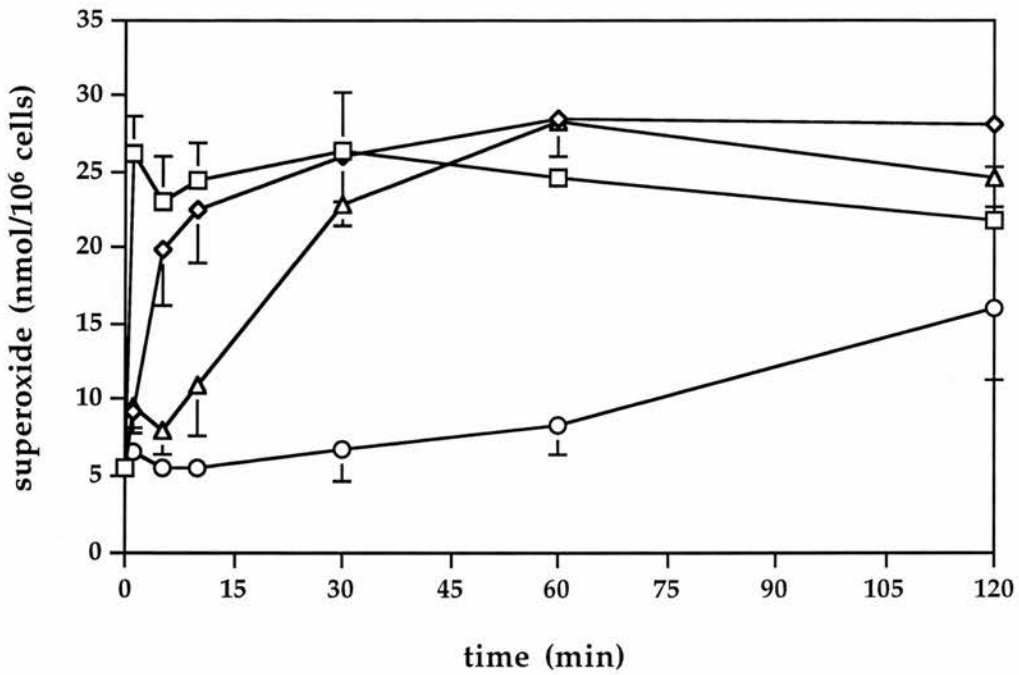


Figure 3.6 Time-course for PAF-, TNF α -, and LPS-induced Priming of fMLP-stimulated O₂⁻ Generation in Human Neutrophils

Human neutrophils (10⁶ in 90 μ l PBS with calcium and magnesium) were incubated for 0-120 min at 37°C with the following priming agents: PAF 100 nM (□), TNF α 200 U/ml (◇), LPS 100 ng/ml (○) and LPS 100 ng/ml + 1% heat-inactivated serum (Δ). At the appropriate times, the incubation volumes were adjusted to 1 ml by the addition of cytochrome C and fMLP (final concentrations 1.2 mg/ml and 100 nM respectively) for 10 min; cells were pelleted at 4°C and the optical density of the supernatants determined at 550 nm. O₂⁻ release was calculated as described in (2.3). Data represent mean \pm sem for n=23 experiments, each performed in duplicate.

Figure 3.7

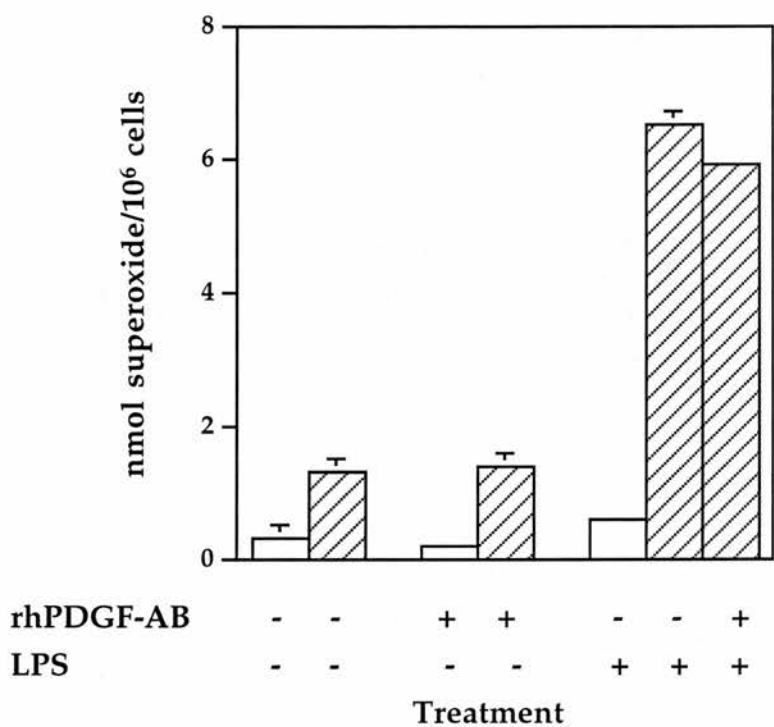


Figure 3.7 The Effects of PDGF on Basal, fMLP-stimulated and LPS-primed Human Neutrophil O_2^- Generation

Human neutrophils (10^6 in 90 μ l PBS with calcium and magnesium) were incubated at 37°C in the presence or absence of recombinant human PDGF-AB (rhPDGF-AB) 20 ng/ml for 30 min; following this period some cells were further incubated with LPS 100 ng/ml for 1 hr. At the end of the designated incubation periods, pre-warmed cytochrome C (final concentration 1.2 mg/ml) and fMLP (100 nM final concentration; hatched bars) or vehicle (open bars) were added, bringing the final incubation volume to 1 ml. Cells were pelleted at 4°C after 10 min, and the release of O_2^- was quantified by measuring the optical density of the supernatants at 550 nm. Data represent the mean \pm sem of 3 separate experiments performed in triplicate.

recombinant human PDGF-AB (which interacts with both α - and β -PDGF receptors) to influence the respiratory burst of human neutrophils. As can be seen from Figure 3.7, PDGF failed to either prime or activate the respiratory burst, and it did not influence the priming response to LPS 100 ng/ml.

3.6 Effects of Inositol Hexakisphosphate on Neutrophil O_2^- Generation

Despite the presence of high concentrations of $InsP_6$ in mammalian cells (up to 15 μ M, Szewergold et al., 1987) its physiological role remains uncertain. Recently it has been reported to act as a neutrophil priming agent (Eggleton et al., 1991); dying and effete cells at a site of inflammation could hence release sufficient quantities of this substance to act as a local mediator of neutrophil function. We therefore sought to validate and enlarge on the original studies. Since $InsP_6$ is acidic in solution, it was dissolved in PBS/25 mM HEPES, which was confirmed to maintain the pH of the reaction medium within the physiological range (7.2-7.4) for $InsP_6$ concentrations of up to 100 μ M. A 2 min preincubation time was selected because previous work in our laboratory has shown that $InsP_6$ 100 μ M for 0.5-2 min significantly augmented fMLP-induced shape change (E. Kitchen, personal communication). In spite of these precautions, $InsP_6$ 100 μ M exhibited only weak priming activity (see Figure 3.8) to both fMLP- and PMA-stimulated O_2^- release; higher concentrations of $InsP_6$ resulted in >80% cell death as assessed by trypan blue positivity. However, in contrast to the modest priming effect of $InsP_6$ alone, incubation of neutrophils with $InsP_6$ for 2 min prior to priming with LPS 100 ng/ml significantly enhanced the effect above that seen with LPS alone (Figure 3.9).

Figure 3.8

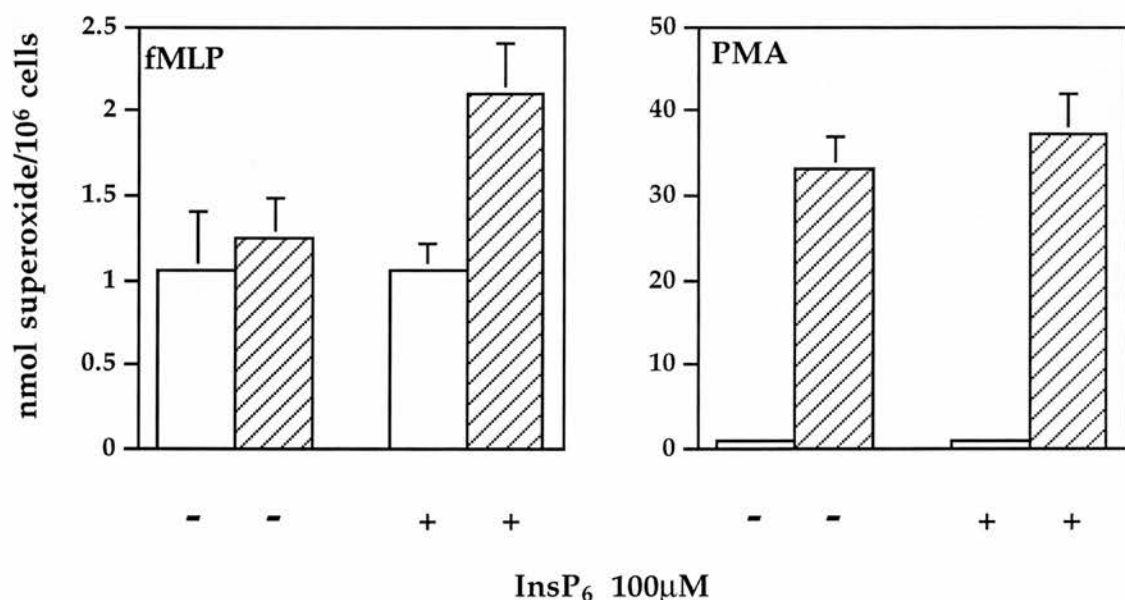


Figure 3.8 Effects of InsP₆ on Human Neutrophil fMLP- and PMA-stimulated O₂⁻ Generation

Pre-warmed human neutrophils (10⁶ in 90 μl PBS with calcium and magnesium) were incubated in the presence or absence of 100 μM InsP₆ (in PBS with 25 mM HEPES) for 2 min at 37°C prior to stimulation with fMLP 100 nM or PMA 10 ng/ml (hatched bars) or the appropriate vehicle control (open bars) in the presence of cytochrome C 1.2 mg/ml (final volume 1 ml) for 10 min. O₂⁻ release was determined by means of the superoxide dismutase-inhibitable reduction of cytochrome C (measurement of the absorbance of the supernatants at 550 nm). Data represent the mean±sem of 6 separate experiments, each performed in triplicate.

Figure 3.9

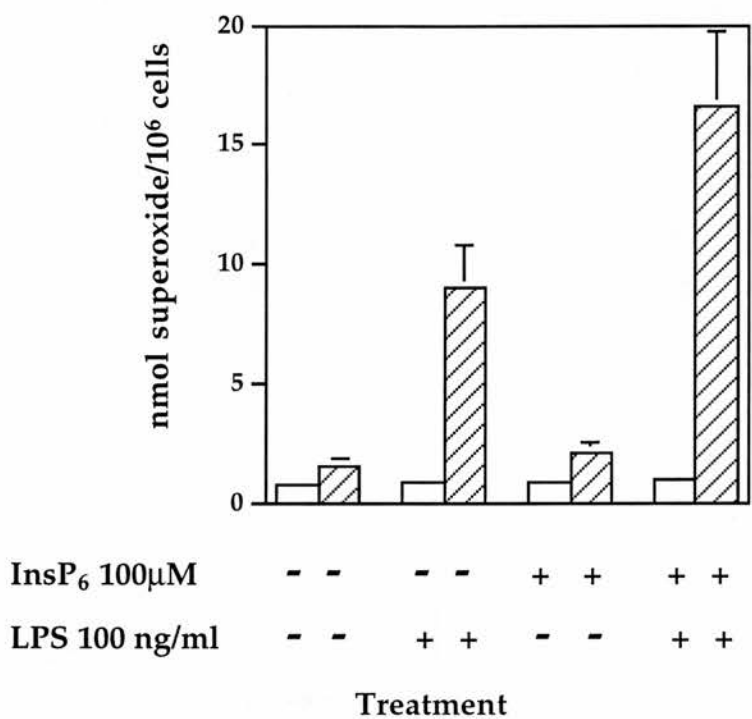


Figure 3.9 Effect of InsP₆ on LPS-induced Priming of fMLP-stimulated O₂⁻ Generation

Pre-warmed human neutrophils (10⁶ in 80 μl) were incubated in the presence or absence of Ins P₆ 100 μM for 2 min prior to the addition of LPS 100 ng/ml or PBS (agonists added in 10 μl) for 60 min. The incubation volume was adjusted to 1 ml by addition of pre-warmed cytochrome C (final concentration 1.2 mg/ml) and fMLP 100 nM (hatched bars) or vehicle (open bars). Superoxide release was determined by the superoxide dismutase-inhibitable reduction of cytochrome C. Data represent the mean±sem of 3 separate experiments, each performed in triplicate.

3.7 Effects of LPS, TNF α and PAF on Human Neutrophil Shape-Change

Neutrophils exposed to activating agents undergo shape-change, initially manifested by protrusion of small pseudopodia, followed by uropod formation and the adoption of a fully polarised morphology. Since it has been suggested that shape-change may correlate with priming (Haslett et al., 1985), we have examined the effects of the three priming agents LPS, TNF α and PAF on shape-change. The results are documented in Figures 3.10-3.11. All three agonists induced shape-change in a concentration-dependent fashion, but the results do not correlate completely with the data obtained for priming of O₂⁻ release; TNF α is more potent than the other agents as an inducer of shape-change, but concentrations of LPS and PAF (10 ng/ml and 10 nM respectively) which have little effect on shape-change will prime for O₂⁻ release (Figure 3.10 versus Figures 3.3 and 3.5).

Figure 3.10

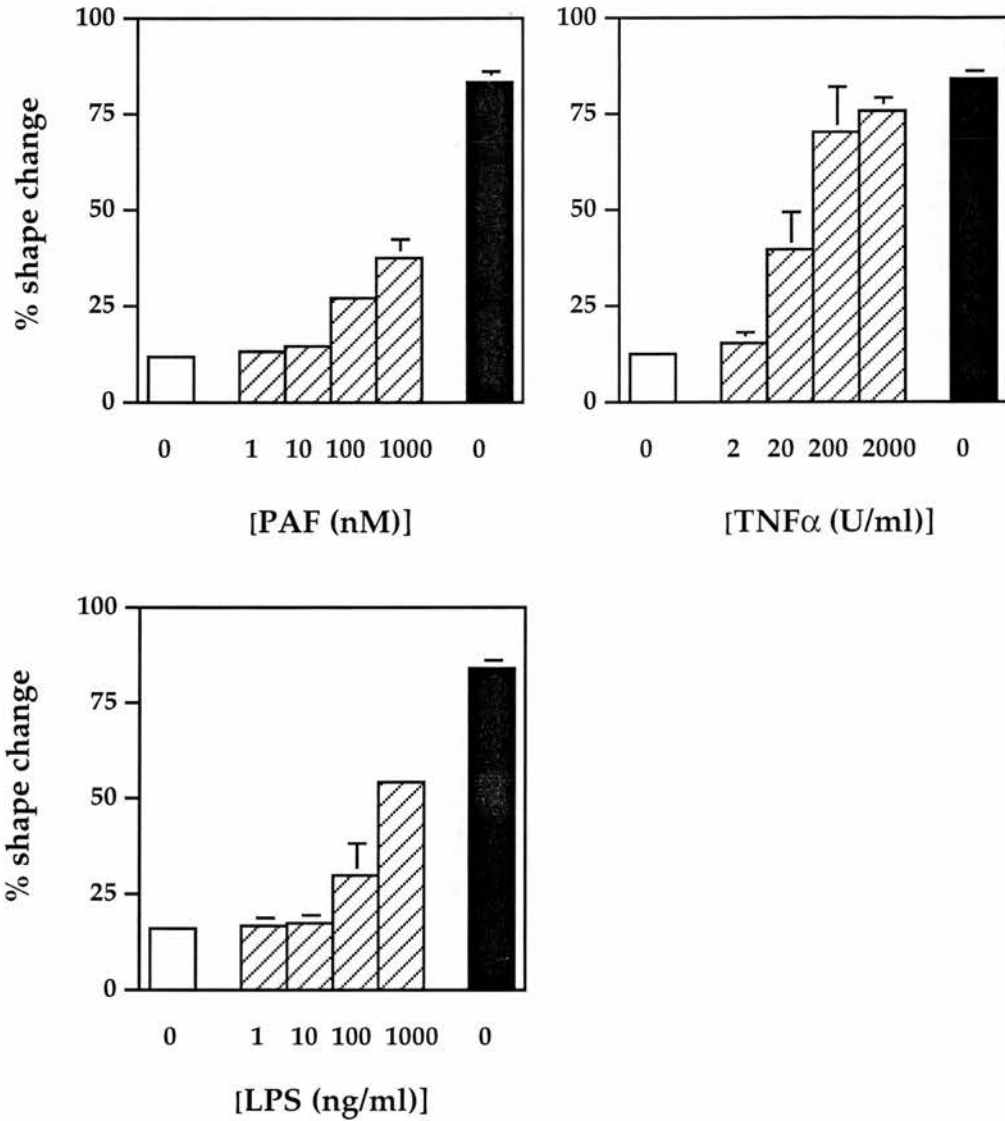
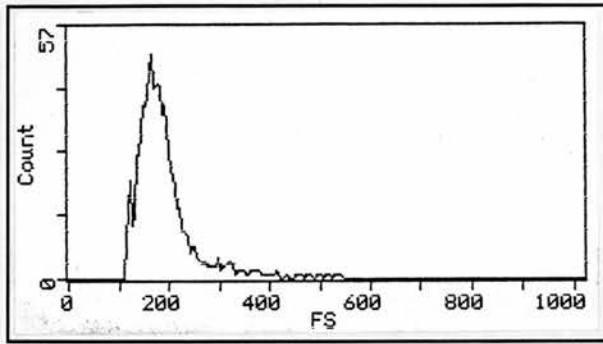


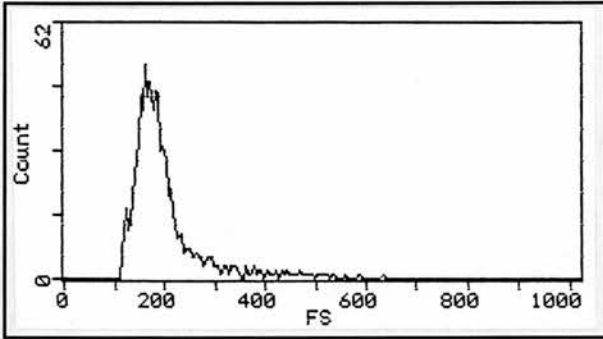
Figure 3.10 Effects of PAF, TNFα and LPS on Neutrophil Shape-Change

Freshly-isolated human neutrophils (5×10^6 in $450 \mu\text{l}$) were incubated at 37°C in the presence of vehicle (open bars), priming agents (PAF $0-1 \mu\text{M}$ (10 min), TNFα $0-2000 \text{ U/ml}$ (30 min), LPS $0-1 \mu\text{g/ml}$ (120 min), hatched bars) or fMLP 100 nM (10 min, closed bars) prior to fixation with $500 \mu\text{l}$ 2.5% glutaraldehyde. Shape-change was assessed by flow cytometry, as described in 2.5. Data represent mean \pm sem for 3 separate experiments, each performed in duplicate.

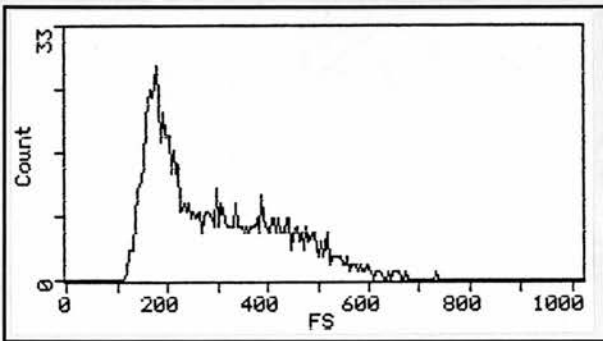
Figure 3.11



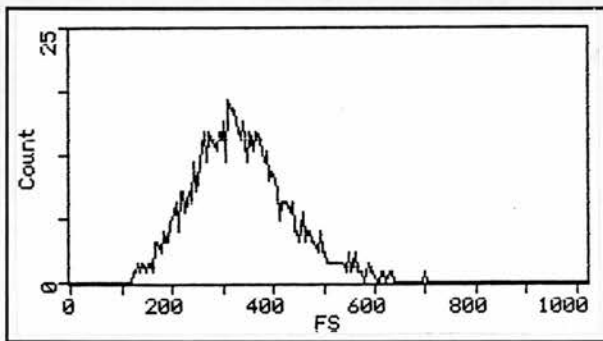
A



B



C



D

Figure 3.11 Effects of TNF on Neutrophil Shape-change
Figure legend overleaf.

Figure Legend for Figure 3.11

Effects of TNF α on Neutrophil Shape-Change

Freshly-isolated human neutrophils (5×10^6 in 450 μ l) were incubated at 37°C in the presence of TNF α 2 U/ml (B), 20 U/ml (C), 200 U/ml (D) or PBS control (A) for 30 min prior to fixation with 500 μ l 2.5% glutaraldehyde. Shape-change was assessed by flow cytometry, as described in 2.5; neutrophil forward scatter (horizontal axis) was plotted against event frequency (vertical axis). Individual plots from a single representative experiment are shown.

3.8 Discussion

The principal aim of the work presented in this chapter was to establish optimum conditions for priming of freshly prepared human neutrophils. As a prerequisite to this aim, a method of neutrophil isolation resulting in minimal basal activation/priming was required. A previous study (Haslett et al., 1985) has demonstrated that exposure to trace amounts of LPS during the separation procedure (LPS was found to be present in the gradient medium Ficoll) can prime neutrophils, and that red cell lysis procedures also affect neutrophil responses; hence we chose to utilise dextran sedimentation and plasma/Percoll gradient separation, a method which obviates the need for a red cell lysis step and which, in the above quoted study, compared favourably with the traditional Ficoll-Hypaque method in terms of the induction of shape change and O_2^- release. The results presented in this chapter demonstrate that we have obtained pure neutrophil populations with minimal basal O_2^- generation and, more importantly, minimal fMLP-induced O_2^- production (invariably ≤ 5 nmol/ 10^6 cells, and usually < 3 nmol/ 10^6 cells, Figures 3.1-3.9). These results compare favourably with those of other authors, whose results may include fMLP-stimulated values of ≥ 10 nmol O_2^- / 10^6 cells (see, for example, Wozniak et al., 1993, Berkow and Dodson 1988, Klein et al., 1995). High values for fMLP-stimulated O_2^- release will 'dilute' the effect of priming agents, reducing the fold-increase in the stimulated respiratory burst seen on priming, and hampering the investigation of the signalling pathways involved. Whilst a theoretical population of totally unprimed human neutrophils might fail to respond to fMLP altogether, this does not seem to be a currently achievable situation, but by using an isolation technique yielding a pure, minimally primed

population of control cells, we were in a strong position to investigate mechanisms of priming.

LPS, TNF α and PAF were all seen to prime neutrophils for O₂⁻ release in a time- and concentration-dependent fashion, consistent with previous results (compare Figures 3.3-3.6 with Table 3.1); higher concentrations of LPS and TNF α activated the NADPH oxidase directly in addition to their priming activity. However, considerable variation in biological activity was noted between different batches of LPS from the same company (for example, compare the efficacy of 100 ng/ml LPS in Figure 3.1A with that illustrated in Figure 3.3); therefore in subsequent experiments all batches of LPS were tested for the ability to prime O₂⁻ release. Since neutrophils remained in contact with serum or PPP until the final two washing procedures during preparation, it is possible that the responses to LPS were influenced by trace concentrations of serum protein (i.e. LBP) not removed by these washes; however, washing procedures were standardised, and consistent results were achieved whilst using each individual batch of LPS. Difficulties were also experienced with variation in the activity of TNF α (U/mg protein, as assessed by cytotoxicity towards mouse L929 cells) varying by > 2 logarithmic orders of magnitude between batches from the same supplier. To circumvent this problem, in agreement with the majority of published literature, TNF α was calibrated in terms of activity (U/ml) rather than in ng/ml. To achieve consistent and reliable priming, the following conditions were selected for use in subsequent experiments: LPS 100 ng/ml for 60-120 min, TNF α 200 U/ml for 30 min and PAF 100 nM for 10 min; similar degrees of priming in the absence of direct activation were regularly obtained under these circumstances.

TNF α exerts its multiplicity of biological effects by interaction with 2 distinct receptors of 55 kDa and 75 kDa (TNF-R55 and TNF-R75 respectively;

Brockhaus et al., 1990). In general, the TNF-R55 is the predominant signalling moiety, while TNF-R75 seems to fulfil an accessory role in enhancing or synergising TNF-R55 effects (Vandenabeele et al., 1995). Richter et al. (1995) demonstrated that both TNF α receptors are involved in the TNF α -induced activation of the respiratory burst of adherent neutrophils, possibly acting in accordance with the ligand-passing model of Tartaglia et al. (1993) whereby TNF α binds first to the high affinity TNF-R75, producing a high local ligand concentration, and then dissociates to bind the TNF-R55 and mediate intracellular signalling. The distinction between cells in suspension and adherent cells is particularly relevant in the case of TNF α , as highlighted by the studies of Nathan et al. (1987, 1989a); it was demonstrated that cells in suspension did not produce H₂O₂ in response to either TNF α or TNF β , but that neutrophils plated on polystyrene surfaces coated with serum, fibronectin, vitronectin, laminin or human umbilical vein endothelial cells (HUVEC), underwent a massive and sustained (70-80 min) secretory response to this cytokine after a lag period of approximately 30 min. This effect was found to be dependent on the engagement of neutrophil integrin receptors (Nathan et al., 1989b) and could be abolished by disruption of microfilament integrity with cytochalasins during the lag period. This phenomenon was not felt to represent priming, however, since its establishment was dependent on the simultaneous rather than consecutive presence of the two 'stimuli' (adherence and the cytokine). Similar findings were reported for GM-CSF and GCSF but not for LPS (Nathan 1989a). These studies emphasise the importance of keeping cells in suspension during the priming period; this was achieved in our experiments by the use of polypropylene Eppendorf tubes and a shaking water-bath operating at 100 horizontal cycles per min. Under these conditions, cells

remained in suspension (macroscopically and microscopically) for periods in excess of 2 hrs.

InsP₆ alone had no effect on basal O₂⁻ release and caused only a very minor (1.8±0.3-fold, n=6) enhancement of fMLP-stimulated O₂⁻ generation; these results are in agreement with those of Eggleton et al. (1991), but it was felt that this effect was too slight to allow InsP₆ to be a useful tool in the study of the mechanisms of priming. The synergy with LPS priming (Figure 3.9) is of interest, since at an inflamed site multiple agonists will be present; in view of the plentiful stores of InsP₆ present in mammalian cells high local concentrations may be achieved at inflammatory foci and this interaction may become physiologically relevant.

PDGF has been reported to influence neutrophil adhesion molecule expression (Garcia-Aguilar et al., 1989); however it has been suggested that many or perhaps all of the effects on neutrophil function previously ascribed to PDGF may be due to contaminants such as β-thromboglobulin and platelet factor-4 (Deuel et al., 1981, Allegrezza-Giulietti et al., 1991), which co-purify with PDGF. In addition, Shure and co-workers (1992) have demonstrated that, despite previous reports (Ferns et al., 1990, Siegbahn et al., 1990), PDGF-AA induced monocyte chemotaxis only in the presence of lymphocytes or IL-1; contaminating mononuclear cells and their products represent a potential source of error in studies where impure neutrophil preparations have been used. In order to circumvent these problems and to clarify the role of PDGF as a mediator of neutrophil function, we have examined the effects of recombinant human PDGF-AB (which activates both PDGF-α and -β receptors) on pure populations of freshly isolated human neutrophils. As demonstrated in Figure 3.7, PDGF had no significant effects on either basal or stimulated O₂⁻ generation. In addition, PDGF failed to influence the priming effects of LPS (Figure 3.7). We have subsequently

shown (Qu et al., 1995) that PDGF does not affect neutrophil shape change, CD11b/CD18 or CD62-L expression, Ins(1,4,5)P₃ accumulation or the phosphorylation of MAPK; and indeed that neutrophils do not possess functional receptors for this peptide. PDGF has therefore been excluded from further studies on neutrophil priming.

fMLP alone induced dramatic shape-change in > 90% of neutrophils (Figures 2.3 and 3.10); exposure to priming agents produced less dramatic shape-change, with formation of small pseudopodia (Stocks et al., 1995). TNF α was the most potent agonist studied in inducing both shape-change and a primed O₂⁻ response (Figures 3.4, 3.10 and 3.11); the correlation with priming activity was not absolute, however, since for LPS and PAF lower concentrations of agonist were required to induce priming than to cause shape-change.

These three agonists utilise differing methods of signal transduction; PAF interacts with a classical G-protein-coupled receptor, whilst binding of TNF α to its receptor induces receptor trimerisation and tyrosine kinase activity. LPS interacts with CD14 to initiate signal transduction; this interaction is promoted by the formation of LPS-LBP complexes but is thought to occur, albeit more slowly, in the absence of serum-derived factors (Shapira et al., 1995). Thus three agonists, acting via different signal transduction pathways, achieve a final common effect (i.e. priming), suggesting a convergence of the transduction pathways. The pathways operative during the induction of priming with these three agents have therefore been further studied in an attempt to define a mechanism/effect common to all priming agents.

In summary, the ideal conditions for priming with LPS, TNF α and PAF within our system have been established by optimisation of the primed, fMLP-stimulated release of O₂⁻. The putative role of PDGF as a mediator of

neutrophil function has been refuted, and InsP_6 has been found to have only modest and transient priming effects (although it does synergise with LPS).

Chapter 4: The Effects of Priming Agents on the Expression and Function of Neutrophil Adhesion Molecules

4.1 Introduction

To reach a site of infection or inflammation, circulating neutrophils must adhere to, and subsequently migrate through the vascular endothelium. Under normal circumstances, the endothelium constitutes a barrier that confines leukocytes to the circulation, although factors such as flow disturbances and (especially) collisions with erythrocytes promote contact with endothelial cells (Schmid-Schönbein et al., 1980). Adjacent to an inflamed focus however, leukocytes roll along the endothelium with reduced velocity, and a proportion of the rolling population becomes arrested, spread and firmly adherent in preparation for migration between endothelial cell junctions. This process is dependent on the co-ordinated and sequential regulation of expression and function of a series of cell adhesion molecules. Inflammatory cytokines render endothelial cells 'stickier' to leukocytes by upregulating the expression of intercellular adhesion molecule-1 (ICAM-1), P-selectin (CD62-P) and E-selectin (CD62-E) (Dustin et al., 1986, Geng et al., 1990, Bevilacqua et al., 1987). Rolling is a reversible, low-affinity interaction mediated by the vascular selectins (CD62-P and CD62-E) and the constitutively-expressed leukocyte antigen CD62-L (reviewed by Bevilacqua and Nelson, 1993, Carlos and Harlan, 1994, Albeda et al., 1994). Research into selectin ligands is ongoing; recently identified native ligands (reviewed by Hogg and Berlin, 1995) include GlyCAM-1, CD34 and MAdCAM-1, which bind CD62-L; PSGL-1, a ligand for CD62-P and probably CD62-E, and a higher-affinity CD62-E ligand, ESL-1. Firm adhesion is principally a function of the leukocyte β_2 -integrins, particularly CD11b/CD18 (Mac-1),

whose expression and function are upregulated by a number of secretagogue agonists (Kishimoto et al., 1989, Jutila et al., 1989). The endothelial counter-receptor for CD11b/CD18 is ICAM-1 (Smith et al., 1989, Dustin et al., 1986); defective or deficient β_2 -integrin expression results in a failure of leukocyte recruitment and recurrent infections (leukocyte adhesion deficiency, LAD; Anderson and Springer, 1987, Kishimoto et al., 1987). As discussed further below (4.7), ligation and clustering of leukocyte integrins leads not only to adhesive events ('outside-in' signalling) but also to cytoskeletal reorganisation and the generation of a series of intracellular signals controlling adhesion-induced changes in cell physiology (reviewed by Clark and Brugge, 1995). Finally, other molecules may have less specific roles; for example, it has been suggested that large, negatively charged glycocalyx molecules such as CD43 (leukosialin) may interfere with receptor-ligand interactions, and may prevent tight contact between large membrane areas due to electrostatic repulsion between negative charges present on the surface of leukocytes (Lichtman and Weed, 1970) and endothelial cells (Pelikan et al., 1979). Shedding of such molecules would be expected to promote adhesion, and in CD43-deficient mice T-cell adhesion has been shown to be enhanced in comparison with that seen in wild-type litter mates (Manjunath et al., 1995).

The actual passage of leukocytes through the endothelial barrier is poorly understood; depending on the inflammatory stimulus and the responding cell, integrins may be involved (Babi et al., 1995, Chuluyan et al., 1993), and shedding of CD62-L seems to precede diapedesis (Kuhns et al., 1995). CD31 (PECAM-1), which is localised to the intercellular junctions between endothelial cells, also appears to be involved, as illustrated by the ability of anti-PECAM-1 antibody to block transmigration (Muller et al., 1993, Vaporciyan et al., 1993). Furthermore, Ca^{2+} fluxes control cell shape at these

junctions, suggesting that endothelial cell signalling can influence the passage of leukocytes (Huang et al., 1993). A schematic representation of leukocyte emigration is shown in Figure 4.1.

It is well recognised that many inflammatory mediators, including certain priming agents, can influence neutrophil adhesive properties by upregulating integrin expression and function and causing shedding of CD62-L (Kishimoto et al., 1989, Jutila et al., 1989); however these studies have focused largely on the effects of high (often supraphysiological) agonist concentrations, with little emphasis on the possible relationship between priming and the regulation of adhesion. The fact that integrins (and possibly also selectins; see section 4.7) have been implicated in signal transduction, possibly synergising with other activating pathways, provided a further inducement to embark on a detailed study of the effects of priming agents (LPS, TNF α and PAF) on the expression and function of neutrophil adhesion molecules.

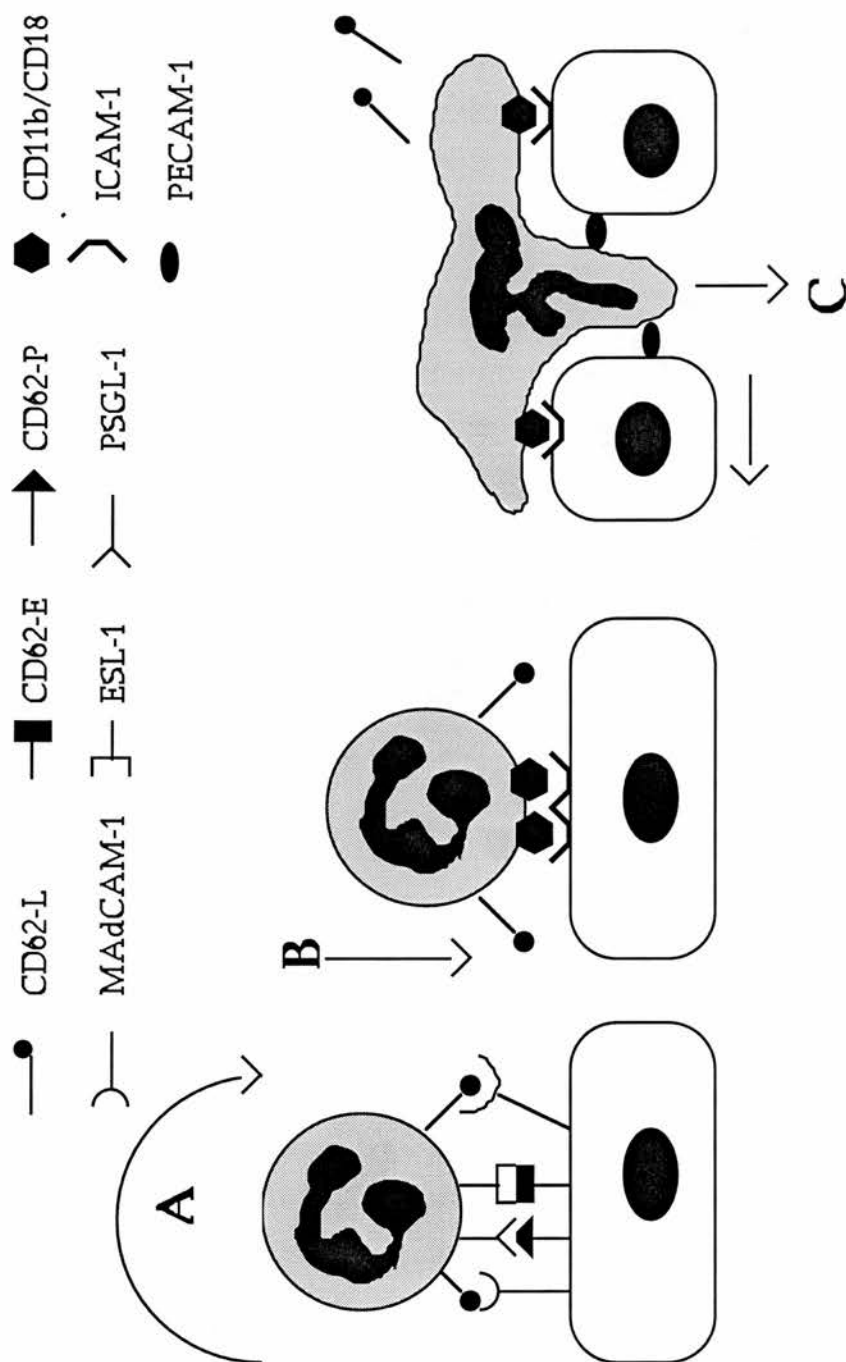


Figure 4.1 A model of Neutrophil Adhesion to the Vascular Endothelium

A. Rolling Adhesion. A transient interaction, mediated by the selectins and their ligands.

B. Firm Adhesion. The neutrophil is tightly bound to the endothelium by integrin-ICAM interactions

C. Transmigration. CD62-L is shed as integrins and PECAM-1 mediate passage between endothelial cells

4.2 Concentration-dependent Effects of LPS, TNF α and PAF on Human Neutrophil Surface Adhesion Molecule Expression

Flow cytometric analysis of human neutrophils incubated with LPS, TNF α or PAF demonstrated that, in common with fMLP, all the priming agents examined caused a concentration-dependent increase in the expression of CD11b, CD11c, and CD35 (Figures 4.2-4.4 and Table 4.1) and reduced CD62-L and, to a lesser extent, CD43 expression (Figures 4.2-4.4); the upregulation of CD11b, CD11c and CD35 paralleled each other closely in all experiments, suggesting similar underlying mechanisms of release. Examination of the concentration-dependency of the above effects revealed important differences in the patterns of receptor modulation induced by the individual agonists. For example, LPS-induced downregulation of CD62-L (EC_{50} 53 \pm 6 ng/ml) occurred at 10 ng/ml (serum-free conditions) in the absence of significant detectable alterations in CD11b (EC_{50} >204 \pm 49 ng/ml; see Figure 4.5), CD11c, CD35 or CD43 expression. In contrast, low concentrations of PAF upregulated CD11b (EC_{50} 38 \pm 12 nM), CD11c and CD35 without any concomitant reduction in the expression of CD62-L (EC_{50} 102.1 \pm 23 nM; see Figure 4.5), whilst TNF α was more nearly equipotent in inducing increased CD11b (EC_{50} 103.8 \pm 25 U/ml), CD11c and CD35 expression and CD62-L loss (EC_{50} 71.4 \pm 24 U/ml). High concentrations of LPS and PAF (1 μ g/ml and 1 μ M respectively) were more potent than fMLP in promoting loss of CD43, while TNF α had little effect on the expression of this antigen.

Thus the pattern and the concentration-dependence of modulation of both integrin and selectin expression appears to differ markedly between priming agents. Since LPS, TNF α and PAF prime the respiratory burst with greatly different kinetics (see Figure 3.6), we next examined the time-course of action of these agents on the expression of two key elements of the adhesion cascade, CD11b and CD62-L.

Figure 4.2

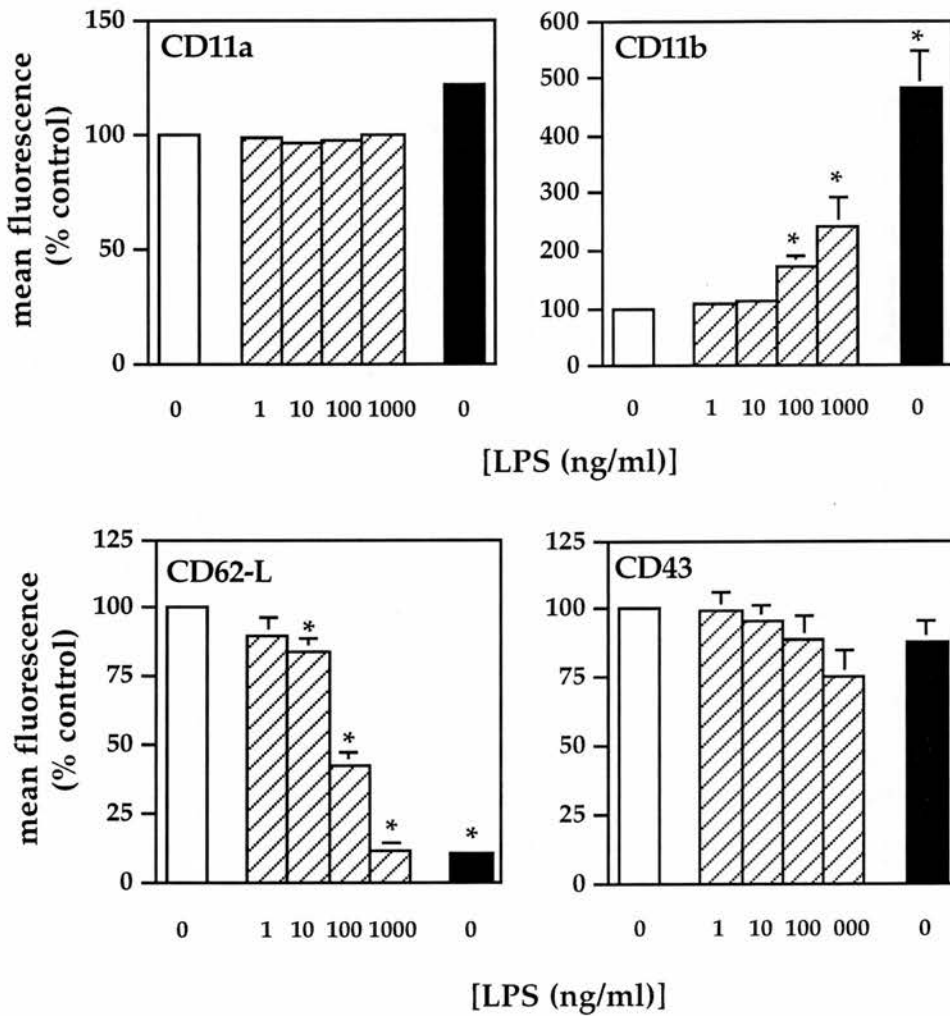


Figure 4.2. Effects of LPS on CD11a, CD11b, CD62-L and CD43 Expression
Neutrophils (5×10^6 /ml) were pre-incubated with (hatched bars) or without (open and closed bars) LPS 1-1000 ng/ml for 60 min. Positive and negative controls received fMLP (final concentration 100 nM, 10 min, closed bars) or vehicle (open bars) respectively. Cells were incubated at 4°C for 30 min with mouse mAb to CD11a, CD11b, CD62-L and CD43 and subsequently with FITC-conjugated rabbit anti-mouse antibody. Mean fluorescence was measured by flow cytometry; data (mean \pm sem, n=5,) are expressed as % of unprimed, unstimulated values. * denotes a significant difference ($p < 0.05$) from control.

Figure 4.3

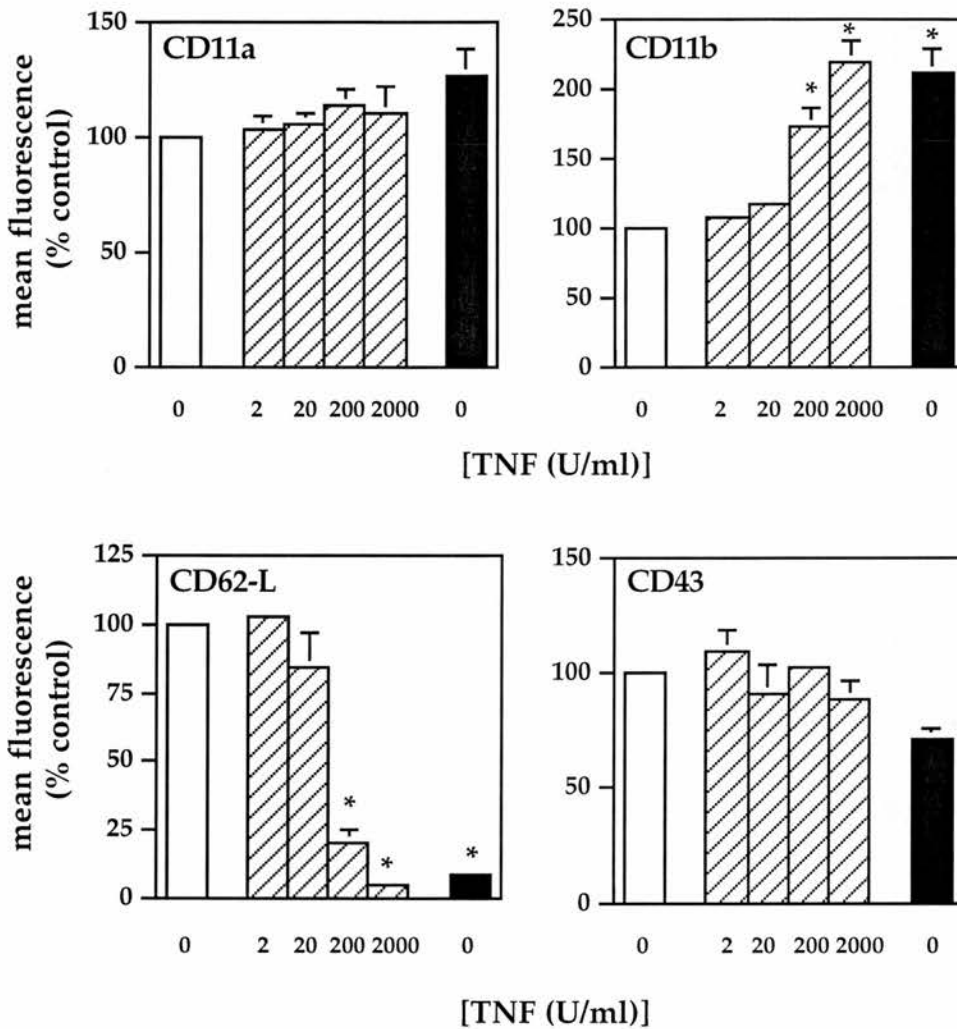
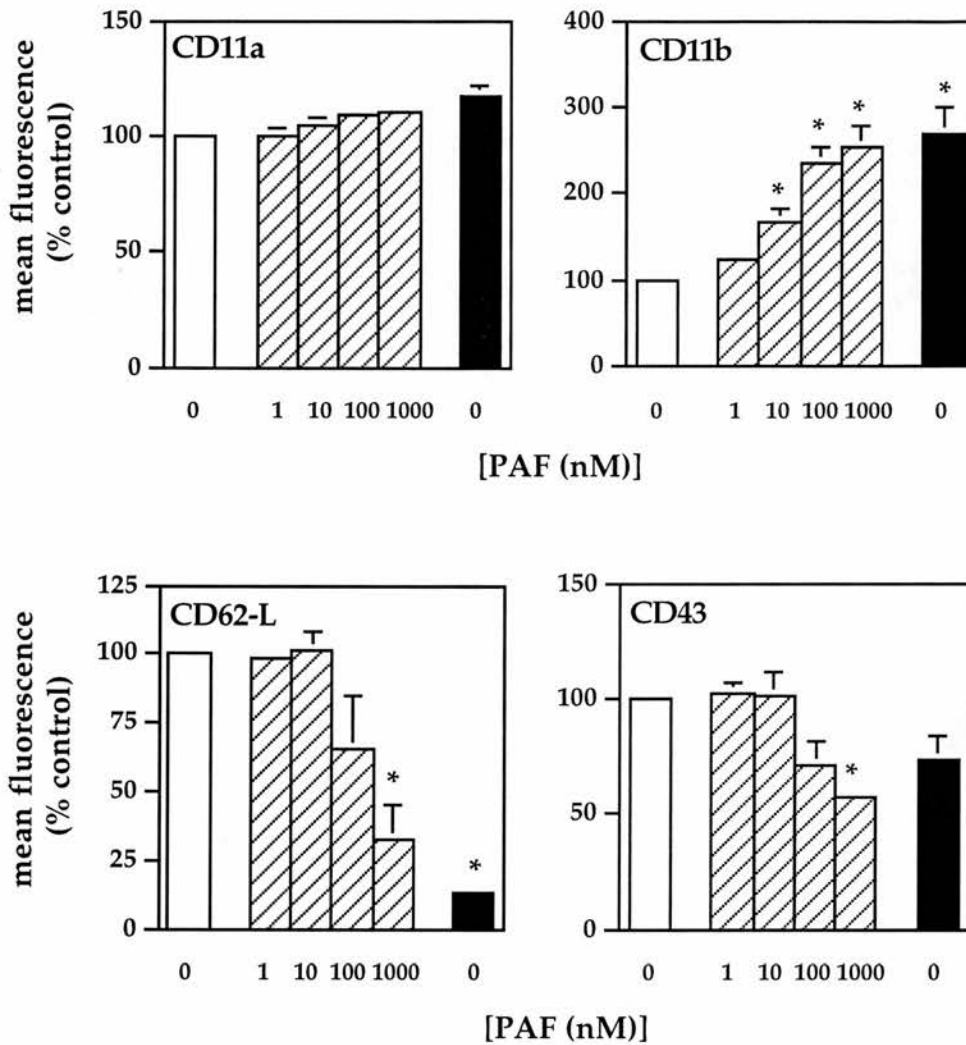


Figure 4.3. Effect of $\text{TNF}\alpha$ on CD11a, CD11b, CD62-L and CD43 Expression Neutrophils ($5 \times 10^6/\text{ml}$) were pre-incubated with (hatched bars) or without (open and closed bars) $\text{TNF}\alpha$ 2-2000 U/ml for 30 min. Positive and negative controls received fMLP (final concentration 100 nM, 10 min, closed bars) or vehicle (open bars) respectively. Cells were incubated at 4°C for 30 min with mouse mAb to CD11a, CD11b, CD62-L and CD43 and subsequently with FITC-conjugated rabbit anti-mouse antibody. Mean fluorescence was measured by flow cytometry; data (mean \pm sem, $n=3$,) are expressed as % of unprimed, unstimulated values. * denotes a significant difference ($p < 0.05$) from control.

Figure 4.4

**Figure 4.4 Effects of PAF on CD11a, CD11b, CD62-L and CD43 Expression**

Neutrophils ($5 \times 10^6/\text{ml}$) were pre-incubated with (hatched bars) or without (open and closed bars) PAF 1-1000 nM for 10 min. Positive and negative controls received fMLP (final concentration 100 nM, 10 min, closed bars) or vehicle (open bars) respectively. Cells were incubated at 4°C for 30 min with mouse mAb to CD11a, CD11b, CD62-L and CD43 and subsequently with FITC-conjugated rabbit anti-mouse antibody. Mean fluorescence was measured by flow cytometry; data (mean \pm sem, $n=3$) are expressed as % of unprimed, unstimulated values. * denotes a significant difference ($p < 0.05$) from control.

Figure 4.5

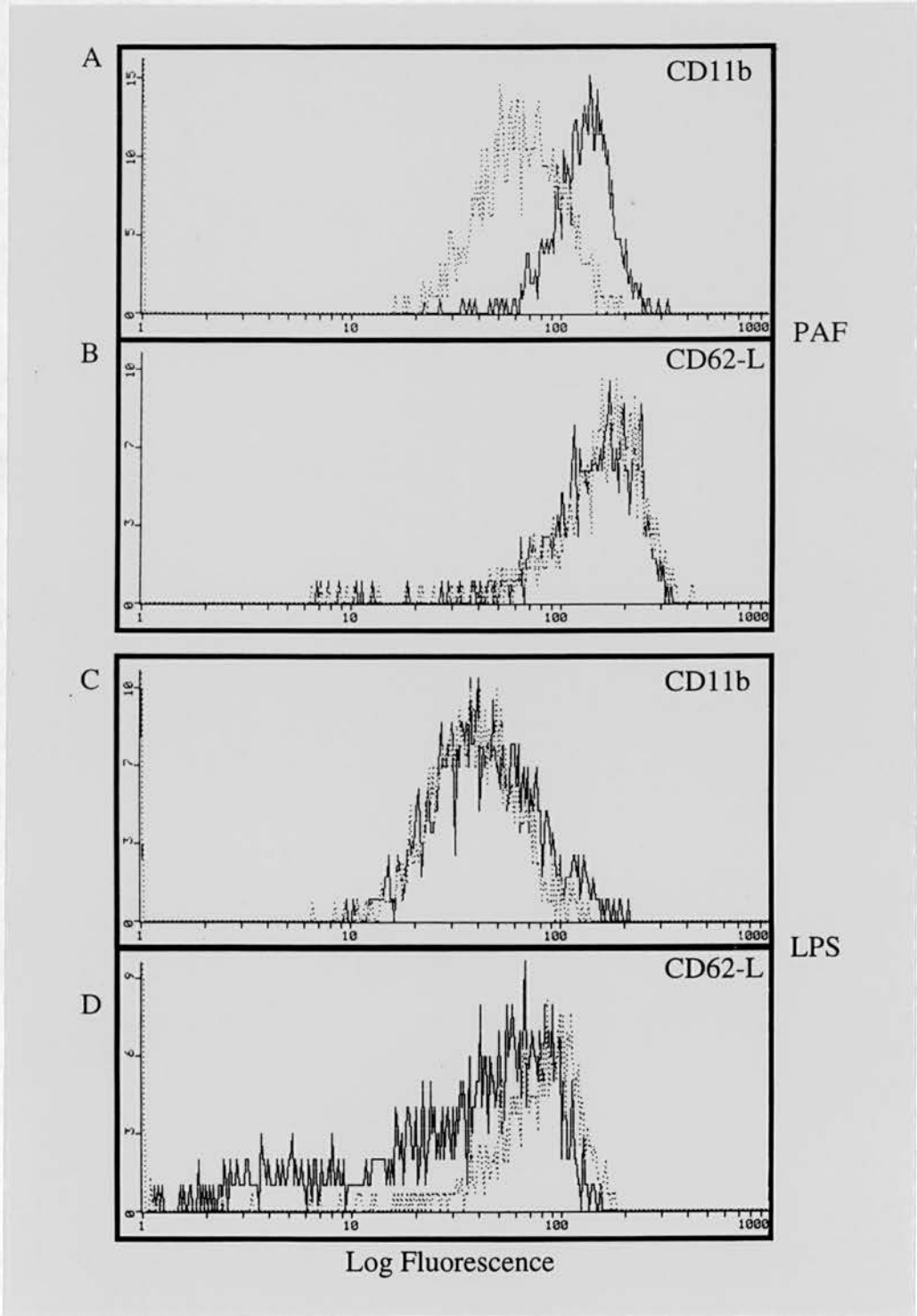


Figure 4.5 Differential Effects of LPS and PAF on Neutrophil CD11b and CD62-L Expression

Figure legend overleaf.

Figure Legend for Figure 4.5

Figure 4.5 Differential Effects of LPS and PAF on Neutrophil CD11b and CD62-L Expression

Neutrophils ($5 \times 10^6/\text{ml}$) were pre-incubated with (A and B) PAF 10 nM (continuous lines) or buffer (stippled lines) for 10 min or with (C and D) LPS 10 ng/ml (continuous lines) or buffer (stippled lines) for 60 min. Cells were subsequently incubated at 4°C for 30 min with mouse mAb to CD11b (A and C) or CD62-L (B and D) and then with FITC-conjugated goat anti-mouse antibody (30 min). Mean fluorescence was measured by flow cytometry. Results from a single representative experiment are shown.

Table 4.1

		CD11c	CD35
LPS (ng/ml)	0	100	100
	1	108.1±6.9	102.1±4.5
	10	104.6±4.5	101.1±5.2
	100	167.4±22.7*	148.2±6.9*
	1000	211.2±14.8*	270.8±6.0*
PAF (nM)	0	100	100
	1	124.0±21.2	118.3±11.0
	10	154.2±29.2	159.8±10.5*
	100	205.6±33.3*	224.3±22.0*
	1000	195.7±35.9*	223.0±34.7*
TNF (U/ml)	0	100	100
	2	105.2±2.1	95.6±4.9
	20	117.5±8.9	102.0±3.6
	200	175.3±13.6*	164.2±8.4*
	2000	199.0±17.8*	233.2±19.7*

Table 4.1 Effects of LPS, TNF α and PAF on CD11c and CD35 Expression

Neutrophils (5×10^6 /ml) were incubated with LPS (60 min), TNF α (30 min) or PAF (10 min) at the concentrations indicated. Cells were then incubated at 4°C for 30 min with mouse mAb to CD11c or CD35, washed and further incubated with FITC-conjugated rabbit anti-mouse antibody. Mean fluorescence was determined by flow cytometry; data (mean±sem, n=3,) are expressed as % of unprimed, unstimulated values. * denotes a significant difference ($p < 0.05$ by Student's paired t-test) from control.

4.3 Time-course of LPS, TNF α and PAF-induced Effects on Human Neutrophil CD11b and CD62-L Expression

To document further the differences between priming agents with respect to surface adhesion molecule expression, the effects of optimal priming concentrations of PAF (100 nM), TNF α (200 U/ml) and LPS (100 ng/ml \pm serum) on CD11b and CD62-L expression were determined over 0-120 min (Figures 4.6 and 4.7).

Incubation with PAF 100 nM produced a rapid (already apparent at 1 min) and profound upregulation of neutrophil CD11b expression, reaching a plateau by 5-10 min (Figure 4.6); this time-course was virtually identical to that seen with 100 nM fMLP (not shown). LPS (100 ng/ml) resulted in a far more gradual increase in CD11b expression, still ongoing at 120 min; this effect was greatly accelerated by the addition of 1% heat-inactivated autologous serum (Figure 4.6) or by increasing the concentration of LPS to 1 μ g/ml (not shown). TNF α 200 U/ml induced CD11b upregulation with a time-course intermediate between PAF and LPS.

Loss of CD62-L was studied in parallel, with similar results being obtained. While CD62-L shedding at 60 min was clearly a very sensitive indicator of LPS-exposure (Figure 4.2), there was a considerable lag before this effect becomes apparent (Figure 4.7); as with the effect on CD11b, the inclusion of serum (or increasing the concentration of LPS, not shown) accelerated CD62-L loss. PAF-induced CD62-L loss, in keeping with its effect on CD11b, was extremely rapid, but less complete than that eventually achieved by other agonists (consistent with the concentration-response data); the effects of TNF α were again intermediate in onset between those seen with LPS and with PAF.

Figure 4.6

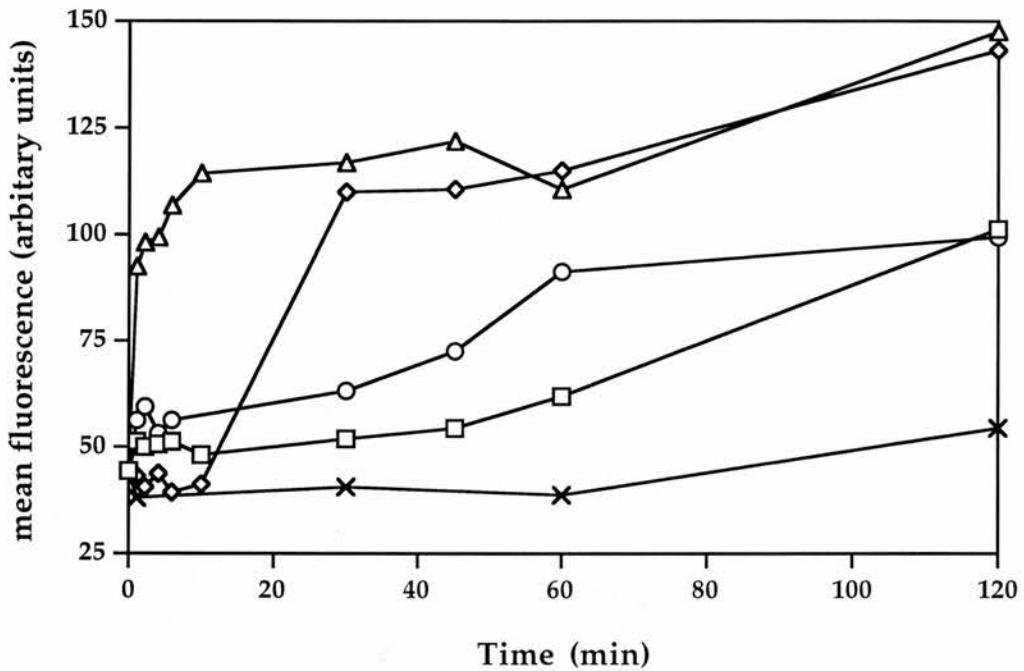


Figure 4.6 Time-course of LPS-, TNF α - and PAF-induced Human Neutrophil CD11b Upregulation

Neutrophils (5×10^6 /ml) were incubated with LPS 100 ng/ml (□), LPS 100 ng/ml + 1% heat-inactivated serum (◇), TNF α 200 U/ml (○), PAF 100 nM (Δ) or 1 % heat-inactivated serum alone (x) for 0-120 min. At the appropriate time-points, cells were transferred to chilled flexiwell (4°C) plates and incubated with mouse mAb to CD11b for 30 min, washed, and incubated with FITC-conjugated rabbit anti-mouse antibody. Mean fluorescence was determined by flow cytometry; data (mean of $n=3$ duplicate measurements, errors (<5%) not shown to preserve clarity) from 1 representative experiment of 5 are shown. PBS alone had no significant effect on CD11b expression (not shown).

Figure 4.7

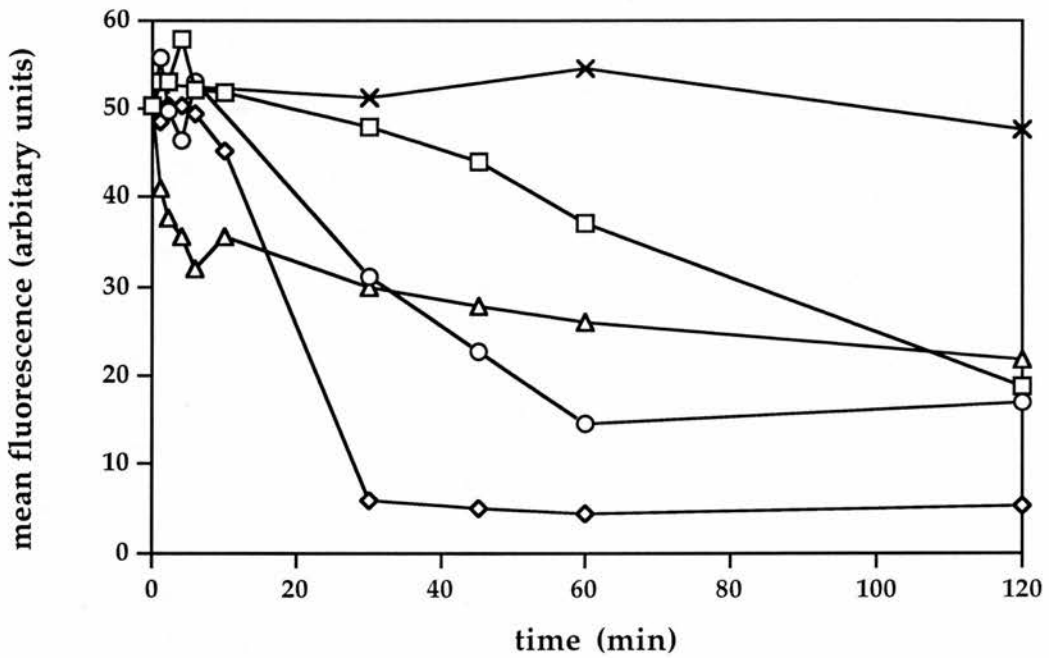


Figure 4.7 Time-course of LPS-, TNF α - and PAF-induced Human Neutrophil CD62-L Loss

Neutrophils (5×10^6 /ml) were incubated with LPS 100 ng/ml (□), LPS 100 ng/ml + 1% heat-inactivated serum (◇), TNF α 200 U/ml (○), PAF 100 nM (Δ) or 1 % heat-inactivated serum alone (×) for 0-120 min. At the appropriate time-points, cells were transferred to chilled flexiwell (4°C) plates and incubated with mouse mAb to CD62-L for 30 min, washed, and incubated with FITC-conjugated rabbit anti-mouse antibody. Mean fluorescence was determined by flow cytometry; data (mean of $n=3$ duplicate measurements, errors (<5%) not shown to preserve clarity) from 1 representative experiment of 5 are shown. PBS alone had no significant effect on CD62-L expression (not shown).

4.4 Effects of LPS, TNF α and PAF on fMLP-induced Modulation of Human Neutrophil Surface Adhesion Molecule Expression

Since priming agents dramatically augment fMLP-induced O₂⁻ release, we sought to determine whether such agonists similarly modulate fMLP-induced changes in surface adhesion molecule expression. Figures 4.8-4.10 demonstrate that incubation of neutrophils with LPS (1-1000 ng/ml), TNF α (2-200 U/ml) or PAF (1-1000 nM) had little influence on subsequent changes in CD11a or CD11b expression induced by fMLP 100 nM; only TNF α 2000 U/ml led to a small but significant potentiation of CD11b detection. However, the higher concentrations of all 3 priming agents (LPS 100 ng/ml and above, TNF α 200 U/ml and above, and PAF 1 μ M) all augmented fMLP-induced CD62-L loss, and to a lesser extent, CD43 loss (Figures 4.8-4.10).

4.5 Effect of LPS, TNF α and PAF on Human Neutrophil Binding of Albumin-coated Latex Beads (ACLB): Concentration-Response

Although only a subset of CD11b/CD18 molecules may mediate adhesion (Diamond and Springer, 1993), newly-recruited integrins may be relevant to adhesion and locomotion (Hughes et al., 1992). Integrin function can be regulated by changes that affect ligand affinity (perhaps secondary to a conformational change, Landis et al., 1993) as well as by altered surface expression. Functional upregulation of CD11b has been shown to result in increased binding of albumin-coated latex beads (ACLB) (Simon et al., 1995); to ascertain whether the above changes in CD11b expression correlate with changes in integrin function, we quantitated the percentage of neutrophils binding FITC-conjugated ACLB after exposure to agonists by flow cytometry (Figures 4.11 and 4.12).

LPS alone increased ACLB-binding only at concentrations of 100 ng/ml and

Figure 4.8

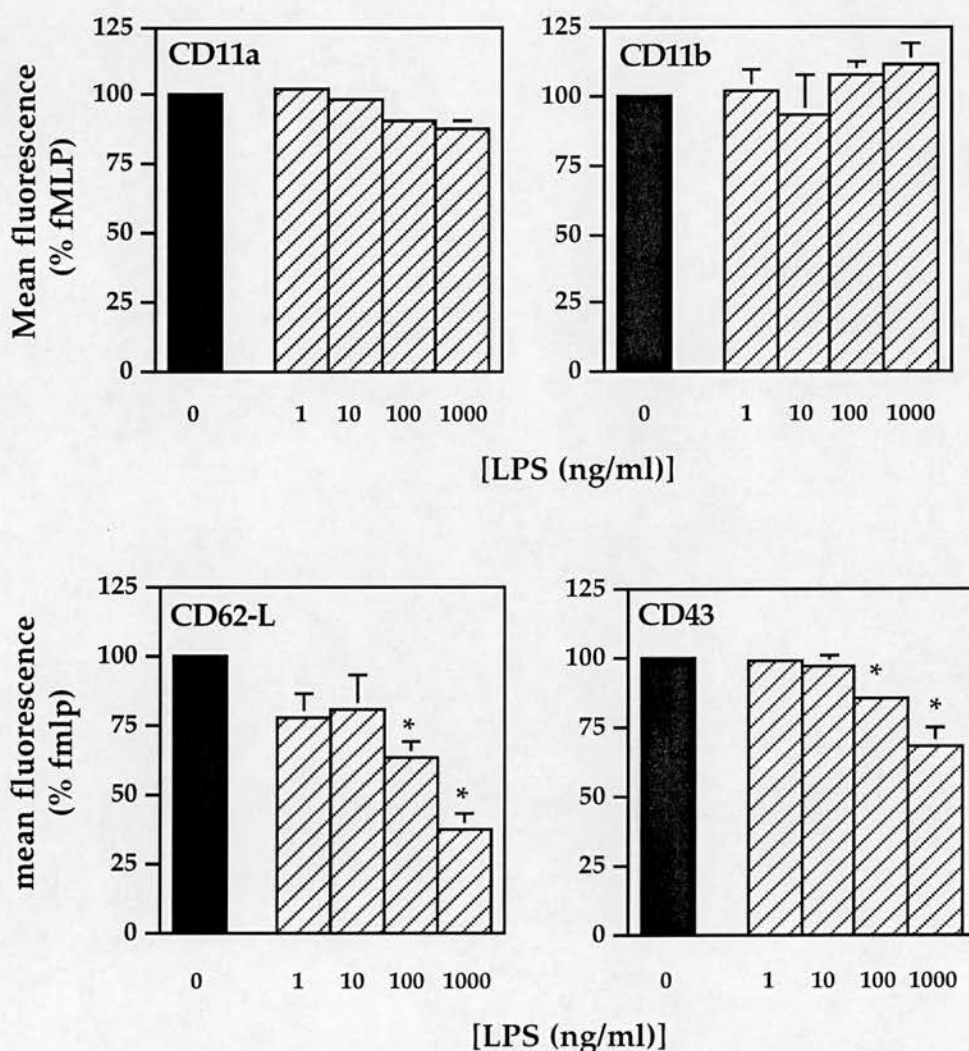


Figure 4.8 Effects of LPS on fMLP-induced Human Neutrophil Adhesion Molecule Expression

Neutrophils (5×10^6 /ml) were incubated with LPS (1 ng-1 μ g/ml) for 60 min (hatched bars) prior to the addition of fMLP (final concentration 100 nM, 10 min). Cells were then treated at 4°C for 30 min with mouse mAb to CD11a, CD11b, CD62-L and CD43, washed and further incubated with FITC-conjugated rabbit anti-mouse antibody. Mean fluorescence was determined by flow cytometry; data (mean \pm sem, $n=5$,) are expressed as % of the fMLP response. * denotes a significant ($p < 0.05$ by Student's paired t-test) difference from fMLP alone (closed bars).

Figure 4.9

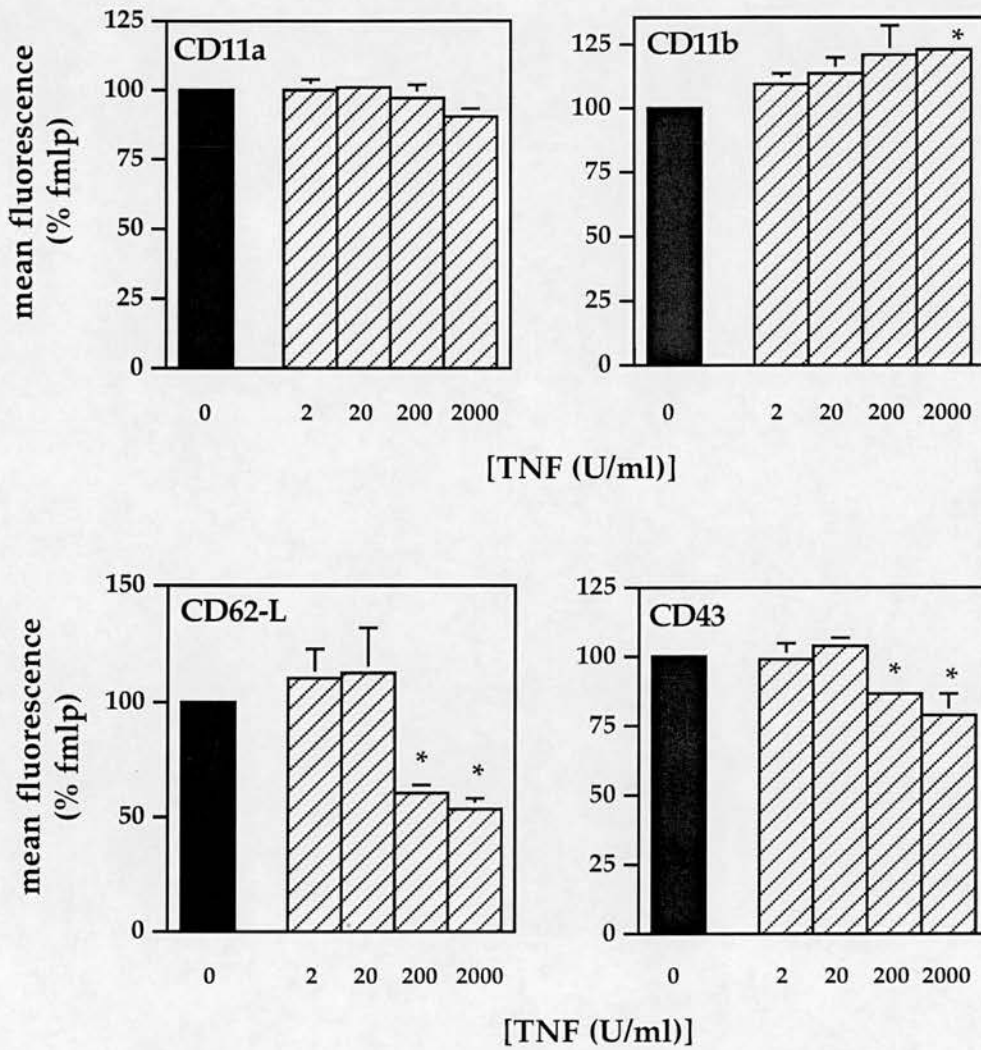


Figure 4.9 Effects of $\text{TNF}\alpha$ on fMLP-induced Human Neutrophil Adhesion Molecule Expression

Neutrophils ($5 \times 10^6/\text{ml}$) were incubated with $\text{TNF}\alpha$ 2-2000 U/ml for 30 min (hatched bars) prior to the addition of fMLP (final concentration 100 nM, 10 min). Cells were then treated at 4°C for 30 min with mouse mAb to CD11a, CD11b, CD62-L and CD43, washed and further incubated with FITC-conjugated rabbit anti-mouse antibody. Mean fluorescence was determined by flow cytometry; data (mean \pm sem, $n=5$,) are expressed as % of the fMLP response. * denotes a significant ($p < 0.05$ by Student's paired t-test) difference from fMLP alone (closed bars).

Figure 4.10

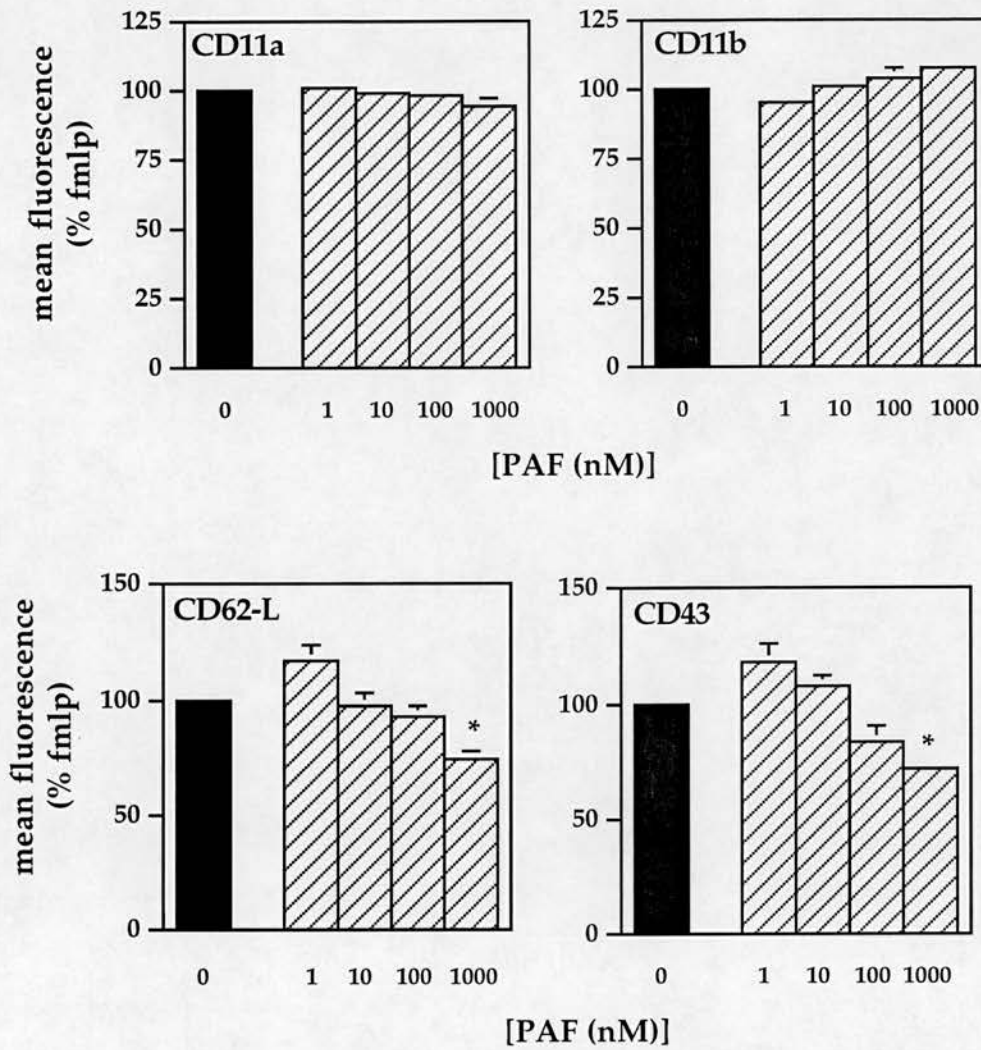


Figure 4.10 Effects of PAF on fMLP-induced Human Neutrophil Adhesion Molecule Expression

Neutrophils ($5 \times 10^6/\text{ml}$) were incubated with PAF 1-1000 nM for 10 min (hatched bars) prior to the addition of fMLP (final concentration 100 nM, 10 min). Cells were then treated at 4°C for 30 min with mouse mAb to CD11a, CD11b, CD62-L and CD43, washed and further incubated with FITC-conjugated rabbit anti-mouse antibody. Mean fluorescence was determined by flow cytometry; data (mean \pm sem, $n=5$,) are expressed as % of the fMLP response. * denotes a significant ($p < 0.05$ by Student's paired t-test) difference from fMLP alone (closed bars).

above ($EC_{50} > 263 \pm 49$ ng/ml); the effect was slightly more pronounced at 120 min than at 60 min (Figure 4.11). In contrast, $TNF\alpha$, PAF and LPS with serum were all highly potent, inducing functional CD11b upregulation even at very low concentrations (EC_{50} 12 ± 3.5 U/ml, 30.8 ± 5.1 nM and 1.8 ± 0.2 ng/ml respectively); in addition, the maximal increases in bead binding were similar to that seen with fMLP 100 nM, compared to the weaker effect of LPS alone.

4.6 Effect of LPS, $TNF\alpha$ and PAF on Human Neutrophil Binding of Albumin-coated Latex Beads (ACLB): Time-course

The time-course for functional upregulation of CD11b closely reflected that for increased surface expression (compare Figures 4.7 and 4.13). The effect of PAF on the binding of ACLB was already considerable at 1 min, reached a maximum at 15 min and subsequently declined (Figure 4.13). $TNF\alpha$ (200 U/ml), and LPS 100 ng/ml in the presence of 1% heat-inactivated serum caused increased binding, delayed in onset compared to PAF, reaching maximum and sustained levels at 30 min. In the absence of serum, LPS alone had little effect until 60 min had elapsed, and an ongoing effect at 120 min. PBS containing 1% serum alone did not increase ACLB-binding above control levels.

Figure 4.11

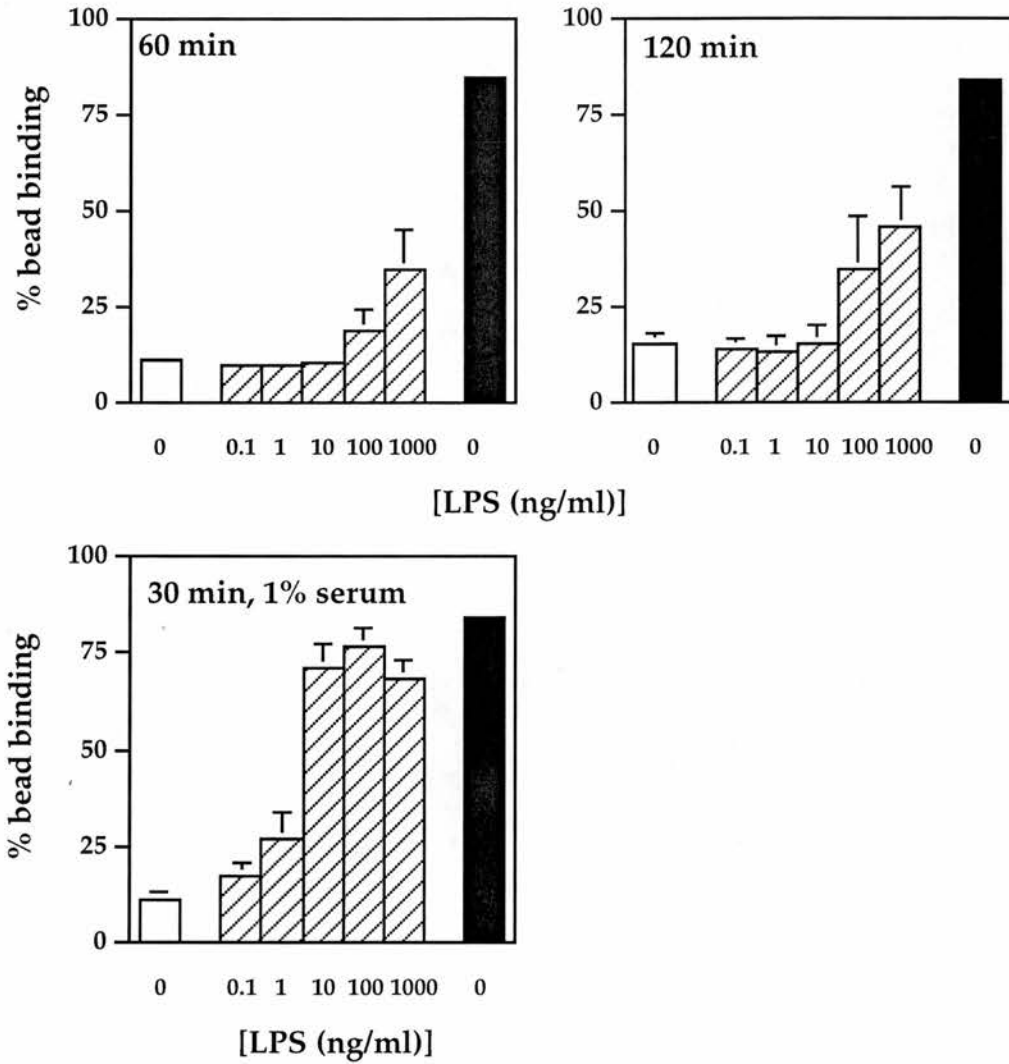


Figure 4.11 Effects of LPS on the Binding of ACLB to Human Neutrophils: Concentration-response

Neutrophils (10^7 /ml, 175 μ l) were incubated with vehicle (open bars), LPS 0.1-1000 ng/ml (hatched bars) alone (60 min and 120 min) or with 1% heat inactivated serum (30 min), or with fMLP 100 nM (10 min, closed bars); 25 μ l ACLB (0.75% in PBS, see 2.5.2) were added 15 min prior to the termination of the incubation (with 0.5 ml 0.5% glutaraldehyde). Cells were subsequently washed three times with PBS to remove non-adherent beads, and the % of neutrophils with adherent beads was determined by flow cytometry. Data represent mean \pm sem of 3 separate experiments each performed in duplicate.

Figure 4.12

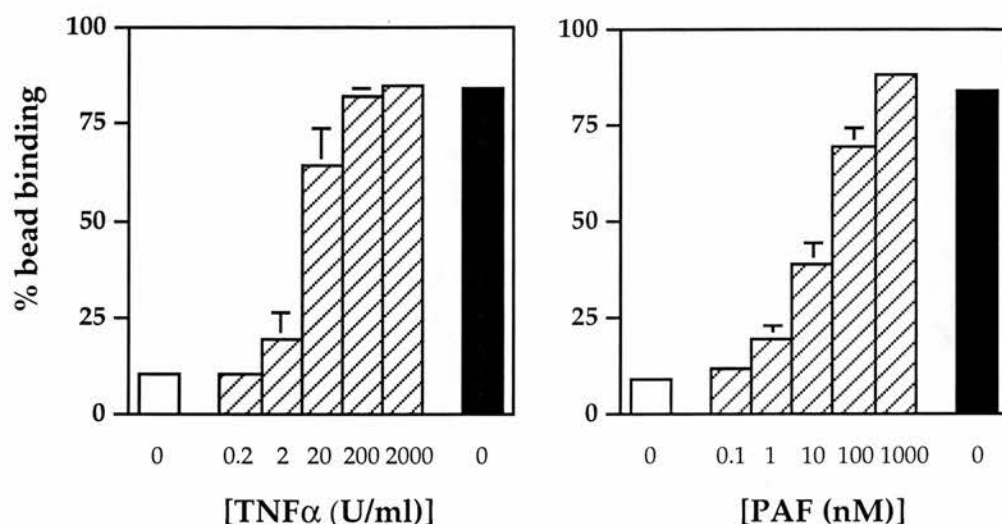


Figure 4.12 Effects of TNFα and PAF on the Binding of ACLB to Human Neutrophils: Concentration-response

Neutrophils (10^7 /ml, 175 μ l) were incubated at 37°C with vehicle (open bars), TNFα (0.2-2000 U/ml, 30 min, hatched bars), PAF (0.1-1000 nM, 10 min, hatched bars) or fMLP (100 nM, 10 min, closed bars). 25 μ l ACLB (0.75% in PBS, prepared as described in 2.5.2) were added 15 min prior to the termination of the incubation (0.5 ml 0.5% glutaraldehyde); for incubations with PAF, ACLB were added 5 min before the agonist. Cells were subsequently washed three times with PBS to remove non-adherent beads, and the % of neutrophils with adherent beads was determined by flow cytometry. Data represent mean \pm sem of 3 separate experiments each performed in duplicate.

Figure 4.13

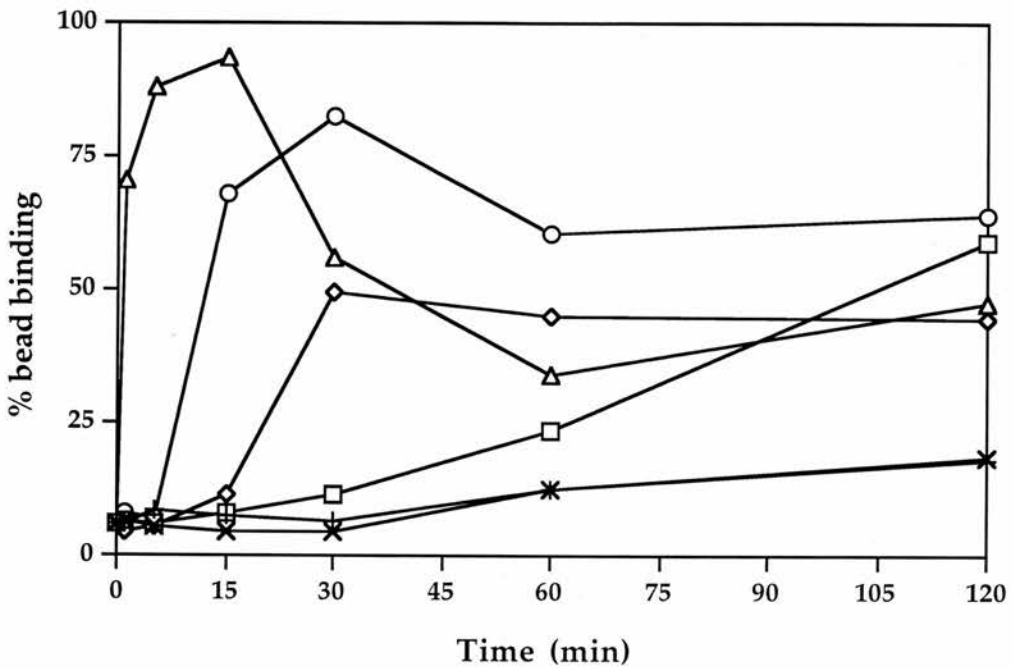


Figure 4.13 Effects of LPS, TNF α and PAF on the Binding of ACLB to Human Neutrophils: Time-course

Neutrophils (10^7 /ml, 175 μ l) were incubated at 37°C with LPS 100 ng/ml with (◇) or without () 1% heat-inactivated autologous serum, TNF α 200 U/ml (o), PAF 100 nM (Δ), 1% heat-inactivated serum alone (x) or PBS alone (+) for the times indicated. 25 μ l ACLB (0.75% in PBS, prepared as described in 2.5.2) were added 15 min prior to the termination of the incubation (0.5 ml 0.5% glutaraldehyde); for incubations of <15 min, ACLB were added before the agonist. Cells were subsequently washed three times in PBS to remove non-adherent beads, and the % of neutrophils with adherent beads was determined by flow cytometry. Data from one representative experiment of 5 are shown. Each point is the mean of 3 determinations; errors (<10%) are not shown to preserve clarity.

4.7 Discussion

The results presented in this chapter have demonstrated that priming agents can modify the expression and function of neutrophil adhesion molecules involved in the trans migratory process at concentrations that do not directly stimulate extracellular O_2^- release as assessed by the cytochrome C reduction assay. To minimise endothelial damage, a transmigrating neutrophil ideally should not be activated, but should be primed for enhanced responsiveness on arrival at the inflammatory focus. The above data suggest that exposure of a marginated intravascular neutrophil to low concentrations of LPS, $TNF\alpha$ or PAF at the inflamed site may modulate the adhesive properties of the cell to promote passage through the endothelium in addition to their priming of secretagogue function. The presence of higher concentrations of these agents within the local vascular space or adjacent to the endothelium could result in full (premature) neutrophil activation and ensuing endothelial damage (Smedley et al., 1986).

Our results showed interesting differences in the regulation of adhesion molecule expression by the different priming agents. For example, while PAF 1-10 nM was sufficient to upregulate CD11b expression and function (and to prime cells for superoxide release, see Figure 3.5), significant loss of CD62-L was only observed with ≥ 100 nM PAF, whilst LPS induced CD62-L shedding at lower concentrations than were required for the upregulation of the β_2 -integrins. Thus there appears to be a dissociation between mobilisation of CD11b/CD18 to the neutrophil surface, a process mediated by exocytosis of specific granules (O'Shea et al., 1985, Stevenson et al., 1987), and the proteolytic shedding of CD62-L (Jung et al., 1990, Jutila et al., 1991). Differential regulation of neutrophil integrins and CD62-L has been reported previously; Molad et al. (1994) reported that occupancy of $Fc\gamma$ receptors by

BSA/anti-BSA immunocomplexes increased expression of CD11b/CD18 without altering CD62-L expression, and Stocks et al. (1995) from our own group described similar findings with antibody to carcinoembryonic antigen; in contrast, preferential loss of CD62-L was observed by Díaz-Gonzalez et al. (1995) in response to a variety of non-steroidal anti-inflammatory drugs. Taken together, these results suggest agonist-specific 'fine-tuning' of neutrophil adhesive behaviour during inflammation.

The kinetics of CD11b upregulation and CD62-L shedding were also very different for the three agents studied, and correlated well with the previously established times required to induce priming (compare Figures 4.6, 4.7 and 4.13 with Figure 3.6). Responses to PAF were in all cases very rapid (observable at 1 min). In the absence of serum, priming and receptor modulation in response to LPS required 60-120 min, while the introduction of 1% serum resulted in these effects being seen at 30 min or earlier (Figures 4.6, 4.7 and 4.13, and Figure 3.6) i.e. with a similar kinetic profile to $\text{TNF}\alpha$. Interestingly, LPS has been shown to induce intracellular PAF accumulation in neutrophils over a time-period similar to that required to establish the primed state (Worthen et al., 1988), suggesting that at least some of the effects of LPS may be indirect. Our results have also demonstrated that integrin function and expression can be dissociated and independently regulated; the effects of PAF on CD11b function (Figure 4.13) declined at later time-points despite sustained levels of expression (compare Figure 4.13 with Figure 4.6).

Adhesion molecules have recently been recognised to participate in signalling events. Integrin receptor engagement and clustering leads to the formation of focal adhesions, with integrins linking to intracellular cytoskeletal components (Otey et al., 1990). Clustering of integrin molecules (induced by ligand-binding) has been shown to elevate intracellular pH

(Schwarz et al., 1991), activate phospholipase C thus elevating intracellular Ca^{2+} and diacylglycerol (and hence activating protein kinase C) (Somogyi et al., 1994), and to stimulate various kinases including focal adhesion kinase (Lipfert et al., 1992) and mitogen-activated protein kinase (MAPK, Cheng et al., 1994). Selectin cross-linking may also transduce messages; intracellular Ca^{2+} release (Laudana et al., 1994), induction of cytokine mRNA (Crockett-Torabi et al., 1995, Laudana et al., 1994) and even potentiation of O_2^- production (Waddell et al., 1994) have all been reported. Whilst the upregulation of neutrophil integrin expression by priming agents could be relevant to signalling in cells adherent to matrix proteins, occupancy of integrin receptors is likely to be limited in cell suspensions, making a major role in the priming system under study unlikely. Since priming agents induce CD62-L shedding rather than cross-linking, this process is also an unlikely candidate for a major role in signalling for the primed state.

Receptor shedding may be required to induce unresponsiveness to extracellular stimuli or to dissociate cells from ligands during the transmigration process; it may also represent a mechanism for the production of soluble receptors which in turn impart signals to other cells (Tedder, 1991). Soluble forms of molecules such as CD62-L are present in normal plasma (Schleiffenbaum et al., 1992), and may be relevant to host defence. For example, in patients at risk of ARDS, a significant association has been found between reduced levels of circulating soluble CD62-L and progression to ARDS (Donnelly et al., 1994). Our data indicate that priming agents may contribute to levels of soluble neutrophil-derived CD62-L, particularly in the vascular micro-environment.

Contemporary models of leukocyte trafficking apply to the systemic circulation, whence neutrophils have been shown to transmigrate principally via the post-capillary venules (Allison et al., 1955, Bjork et al., 1985).

However, the current paradigms cannot be applied to the lung, since the principle site of leukocyte emigration appears to be the pulmonary capillaries (Downey et al., 1993). Given the marked differences between these two vascular compartments (e.g. lower intravascular pressure in the pulmonary circulation, pulsatile flow in the pulmonary capillary bed, and the smaller diameter of the pulmonary capillaries) there will always be close apposition of neutrophils to the pulmonary vascular endothelium, with ample time for interactions (the median transit time of neutrophils through the pulmonary capillary bed has been estimated at 120 sec by Hogg et al., 1994). Thus processes controlling neutrophil migration may have different effects at these two sites; for example, neutrophil adherence in the pulmonary circulation can occur by either CD18-dependent or -independent mechanisms, according to the inflammatory stimulus (Doerschuk et al., 1990; Hellewell et al., 1994).

In summary, priming concentrations of LPS, TNF α and PAF induced agonist-dependent and dissociable changes in human neutrophil adhesion molecule expression and function; the time-courses of these alterations paralleled those for the induction of priming. These data provide strong support for the hypothesis that modulation of adhesion molecule expression may constitute an important mechanism by which priming agents modify granulocyte recruitment as well as granulocyte responses to activation.

Chapter 5. The Role of the G-Protein $G_{i\alpha 2}$ in Neutrophil Priming

5.1 Introduction

Diverse extracellular signals including hormones, growth factors, neurotransmitters and primary sensory stimuli (photons, chemical odorants) all utilise a signal transduction system first identified by Rodbell et al. (1971) consisting of 3 components: receptor, G-protein and effector. The heterotrimeric G-proteins are made up of α -, β - and γ -subunits and function to couple the serpentine or seven transmembrane receptors (including most neutrophil chemoattractant receptors) to downstream signalling pathways (Neer, 1995). More than 200 receptors, plus at least 21 α -subunits, 4 β -subunits and 6 γ -subunits have been identified (Hepler and Gilman, 1992). Although the same $\beta\gamma$ -subunit complex can associate with different α -subunits to form the heterotrimer, the identity of the α -subunit is currently used to define an individual G-protein oligomer.

The α -subunits can be divided into 4 classes: G_s , G_i , G_q and G_{12} ; neutrophils express members of the G_s (Bokoch, 1987), G_i (Goldsmith et al., 1987, Uhing et al., 1987) and $G_{\alpha 12}$ (Strathmann and Simon, 1991) classes, and also unique pertussis toxin-insensitive G-proteins from the G_q class such as $G_{\alpha 15}$ and $G_{\alpha 16}$ (Wilkie et al., 1991 Amatruda et al., 1991), which may mediate activation of PLC β (Smrcka et al., 1991). The identities of the β - and γ -subunit isoforms expressed in neutrophils are currently unknown.

Binding of ligand to a seven transmembrane receptor leads to G-protein activation manifested by an increase in the amount of bound GTP and leading to dissociation of the α - and $\beta\gamma$ -subunits (see 1.8.2). Traditionally, the α -subunit has been regarded as the 'business end' of a G-protein, because

it binds and hydrolyses GTP and interacts with downstream effectors; the $\beta\gamma$ -subunit was previously considered to act solely as a membrane anchor for the heterotrimer. However, a growing body of evidence now supports the idea that free $\beta\gamma$ -subunits can interact functionally with effectors, including the muscarinic cholinergic receptor-regulated K^+ channel in the heart (Logothetis et al., 1987) and an unidentified PLC isoform in neutrophil-differentiated HL-60 cells (Camps et al., 1992).

The effects of certain priming agents on neutrophil (or HL-60 cell) G-protein translocation and function have been investigated; all of the quoted studies have used a single priming agent, and all but one have examined a single time-point. Klein et al. (1992) first demonstrated that a 24 hr incubation with interferon- γ enhanced both detection of $G_{i\alpha 2}$ and $G_{i\alpha 3}$ and GTPase activity in HL-60 membranes; however, studies in HL-60 cells cannot automatically be extrapolated to neutrophils, and there is some doubt as to whether interferon- γ is a true neutrophil priming agent (Pabst, 1994). Yasui et al. (1992) showed that LPS at a concentration of 10 ng/ml in the presence of 1% serum increased the amount of $G_{i\alpha 2}$ detected in neutrophil membranes at 45 min; identical incubation conditions primed the neutrophil respiratory burst, but no other conditions were examined. A study by Durstin et al. (1993) revealed that GM-CSF-induced translocation of $G_{i\alpha 2}$ (but not $G_{i\alpha 3}$) was significant at 5 min and maximal by 40 min, but no priming data was included. Finally, Klein et al. (1995) demonstrated that incubation of neutrophils with $TNF\alpha$ (100 U/ml for 10 min) increased the detection of membrane-associated $G_{i\alpha 2}$ and $G_{i\alpha 3}$, and confirmed enhanced G-protein activity on fMLP-stimulation. The aim of the work presented in this chapter was to extend these studies by examining the relationship between G-protein translocation/activation and neutrophil priming induced by LPS, $TNF\alpha$ and PAF.

5.2 Time-course of Neutrophil Plasma Membrane $G_{i\alpha 2}$ Upregulation in Response to PAF, $TNF\alpha$ and LPS

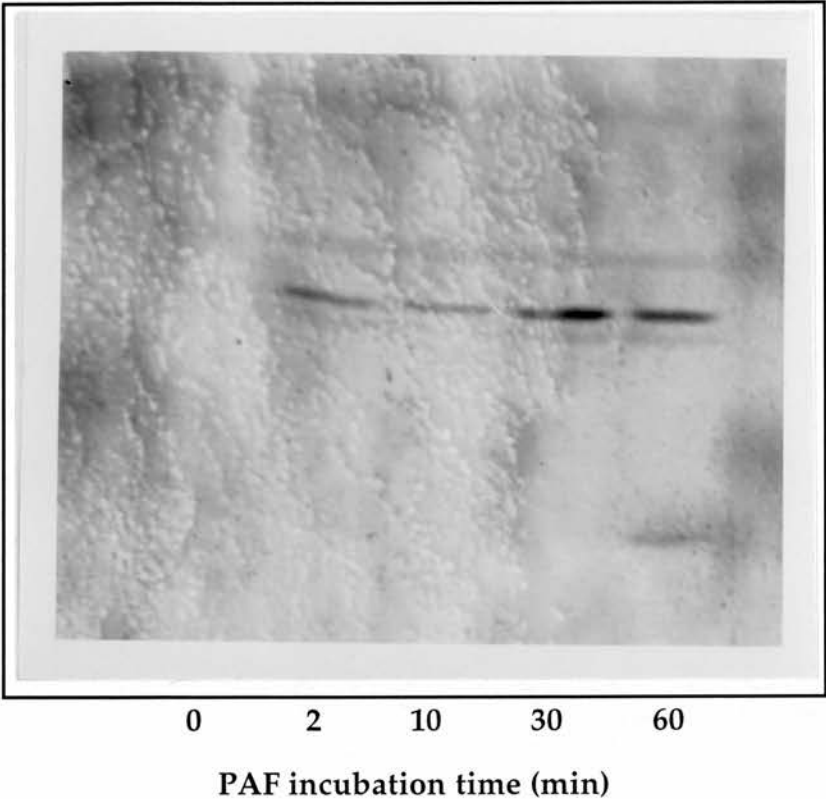
In preliminary experiments, $G_{i\alpha 2}$ detection in isolated, untreated neutrophil plasma membranes was found to be stable with time; therefore, in view of the limited availability of membrane protein, time-matched control samples were not analysed in these experiments. All three priming agents increased the amount of $G_{i\alpha 2}$ detected in equivalent neutrophil plasma membrane fractions, but the kinetics of this effect were agonist-dependent (Figures 5.1-5.3). For example, 100 nM PAF induced a maximal response (of greater magnitude than that seen with the other agents) within 2 min (Figure 5.1) whilst an incubation of 120 min was required to induce a significant response to LPS 100 ng/ml in the absence of serum (Figure 5.3). A 10 min exposure to $TNF\alpha$ 200 U/ml did cause an increase in plasma membrane $G_{i\alpha 2}$, but this was sub-maximal with a further increase becoming apparent by 60 min (Figure 5.2). Comparison with the respective times required to induce priming of agonist-stimulated O_2^- release (see Figure 3.6) revealed very similar agonist-dependent time-courses.

5.3 Concentration-dependence of Neutrophil Plasma Membrane $G_{i\alpha 2}$ Upregulation in Response to PAF, $TNF\alpha$ and LPS

Even very low concentrations of PAF (1 nM) induced a major upregulation of membrane $G_{i\alpha 2}$ expression, with increasing effect up to 1 μ M (the highest concentration examined). A similar concentration-dependent increase in membrane-associated $G_{i\alpha 2}$ was observed with $TNF\alpha$ and LPS, although the degree of upregulation was less marked with these two agents even when the incubation times for all three agonists were selected to obtain a maximal response. Most importantly, comparison of these findings with the

Figure 5.1

A.



B.

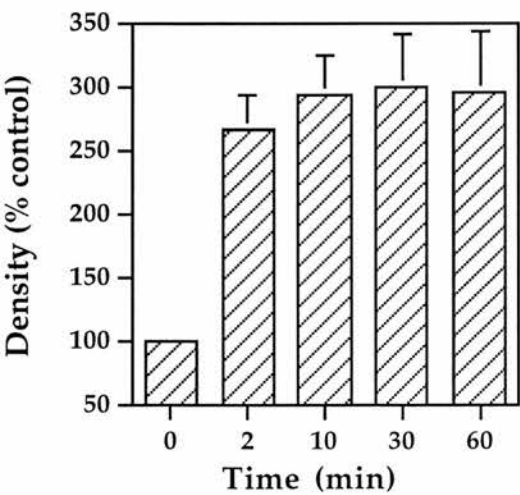
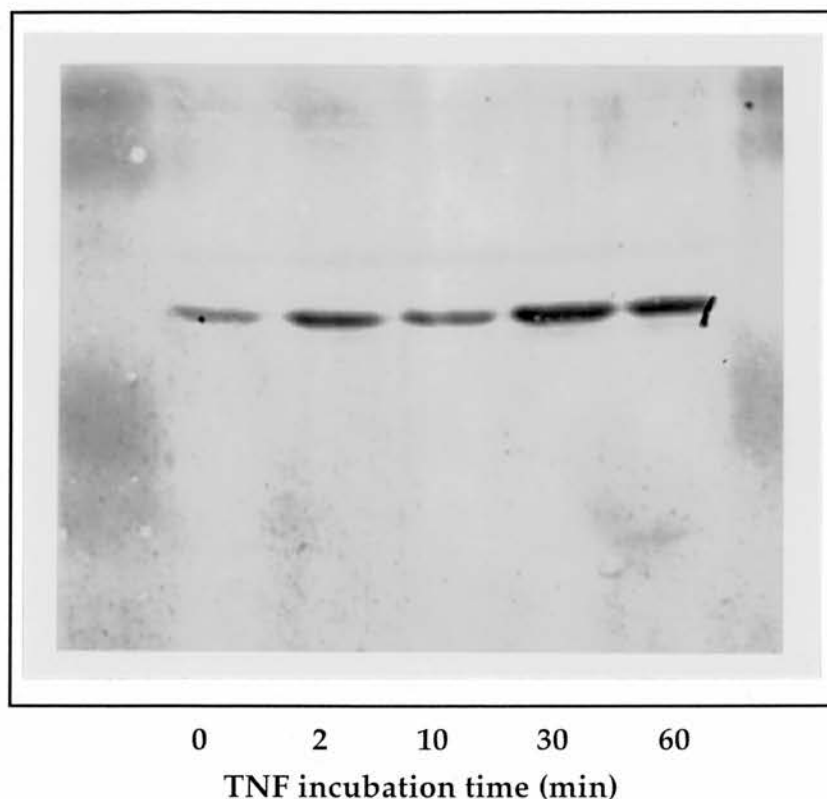


Figure 5.1 Time-course of Neutrophil Plasma Membrane $G_{i\alpha 2}$ Upregulation in Response to PAF

For Figure Legend see page 126.

Figure 5.2

A.



B.

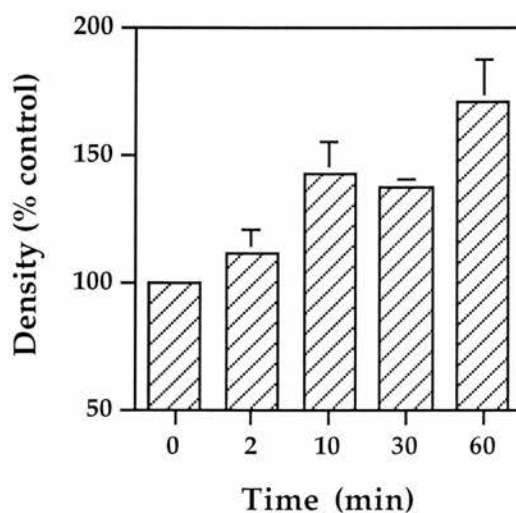
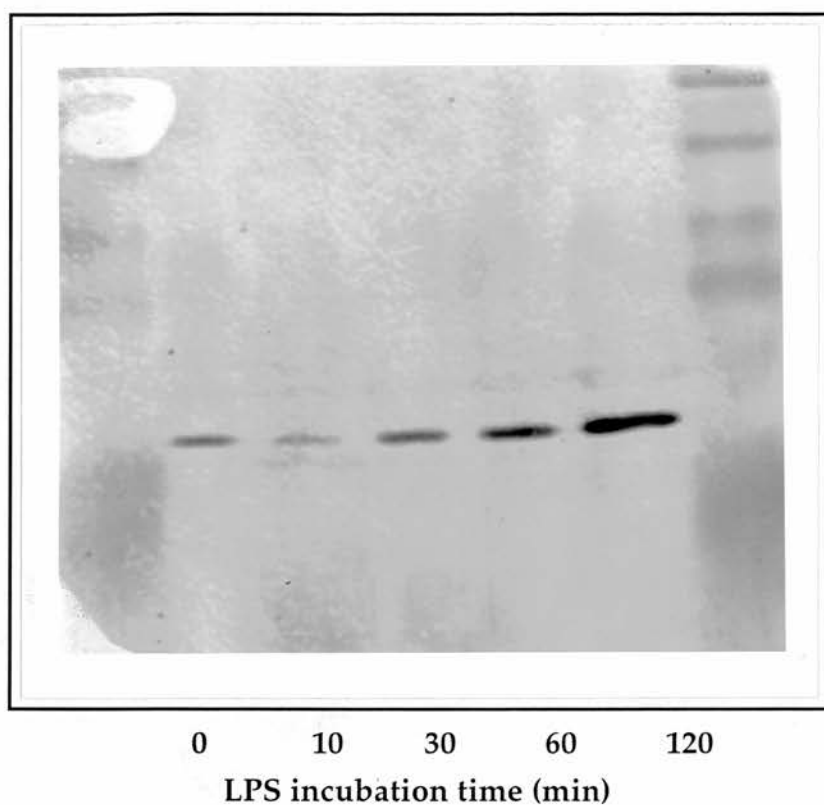


Figure 5.2 Time-course of Neutrophil Plasma Membrane $G_{i\alpha 2}$ Upregulation in Response to $TNF\alpha$

For Figure Legend see page 126.

Figure 5.3

A.



B.

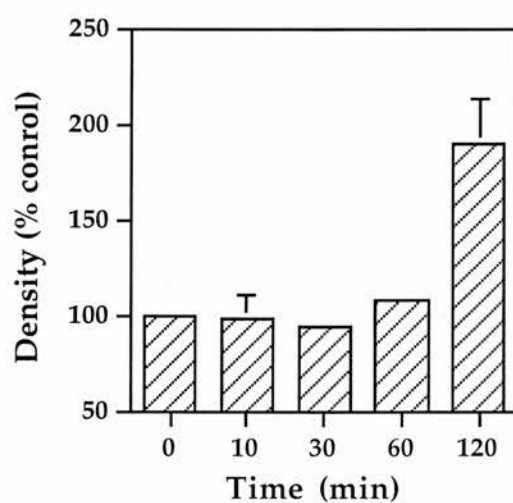


Figure 5.3 Time-course of Neutrophil Plasma Membrane $G_{i\alpha 2}$ Upregulation in Response to LPS

For Figure legend see page 126.

Figure Legends for Figures 5.1-5.3

Figure 5.1 Time-course of Neutrophil Plasma Membrane $G_{i\alpha 2}$ Upregulation in Response to PAF

Human neutrophils incubated with PAF 100 nM for 0-60 min were disrupted by nitrogen cavitation; the plasma membrane fraction was separated by Percoll gradient centrifugation, and analysed by immunoblotting for $G_{i\alpha 2}$ (as described in sections 2.6.1 and 2.6.2). A single representative immunoblot (A) and mean \pm sem densitometry data from n=3 experiments (B) are shown.

Figure 5.2 Time-course of Neutrophil Plasma Membrane $G_{i\alpha 2}$ Upregulation in Response to $TNF\alpha$

Human neutrophils incubated with $TNF\alpha$ 200 U/ml for 0-60 min were disrupted by nitrogen cavitation; the plasma membrane fraction was separated by Percoll gradient centrifugation, and analysed by immunoblotting for $G_{i\alpha 2}$ (as described in sections 2.6.1 and 2.6.2). A single representative immunoblot (A) and mean \pm sem densitometry data from n=3 experiments (B) are shown.

Figure 5.3 Time-course of Neutrophil Plasma Membrane $G_{i\alpha 2}$ Upregulation in Response to LPS

Human neutrophils incubated with LPS 100 ng/ml for 0-120 min, were disrupted by nitrogen cavitation; the plasma membrane fraction was separated by Percoll gradient centrifugation, and analysed by immunoblotting for $G_{i\alpha 2}$ (as described in sections 2.6.1 and 2.6.2). A single representative immunoblot (A) and mean \pm sem densitometry data from n=3 experiments (B) are shown.

Figure 5.4

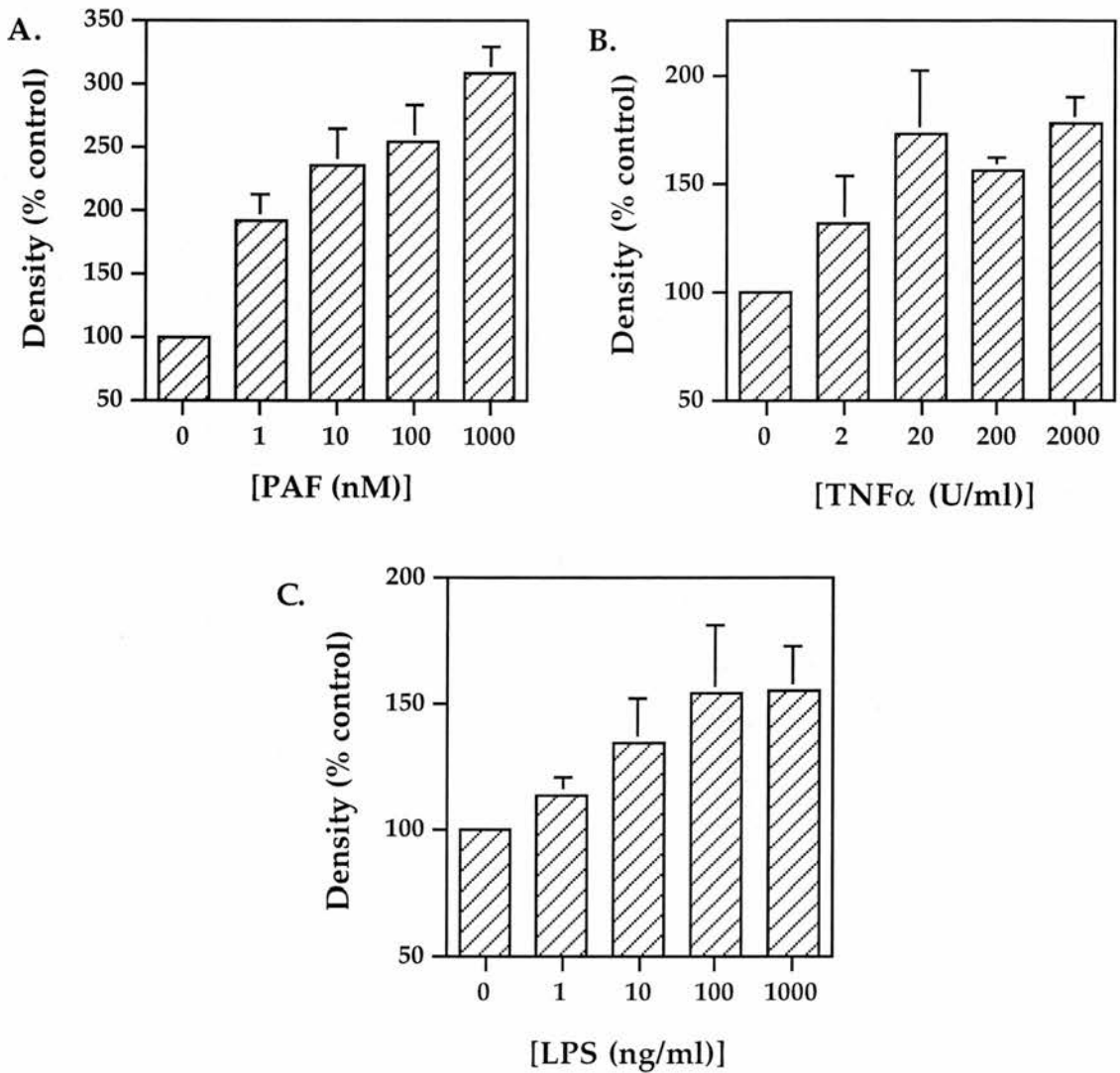


Figure 5.4 Upregulation of Neutrophil Plasma Membrane $G_{i\alpha 2}$ in response to PAF, $TNF\alpha$ and LPS: Concentration-dependence

Human neutrophils incubated with PAF 0-1000 nM (10 min), $TNF\alpha$ 0-2000 U/ml (30 min) or LPS 0-1000 ng/ml (120 min) were disrupted by nitrogen cavitation; the plasma membrane fractions were separated by Percoll gradient centrifugation, and analysed by immunoblotting for $G_{i\alpha 2}$ (sections 2.6.1 and 2.6.2). Results represent mean \pm sem densitometry data from $n=3$ experiments for each priming agent.

concentration-response data for O_2^- priming (Figures 3.3-3.5) reveals that increases in $G_{i\alpha 2}$ stimulated by PAF and $TNF\alpha$ clearly occur at lower agonist concentrations (EC_{50} for LPS, $TNF\alpha$ and PAF 58.1 ± 18.2 ng/ml, 3.4 ± 2.6 U/ml and 3.7 ± 1.8 nM respectively) than those which augment O_2^- release (EC_{50} for LPS, $TNF\alpha$ and PAF 35 ± 14 ng/ml, $>216\pm 48$ U/ml and $>24\pm 16$ nM respectively), and the magnitude of the $G_{i\alpha 2}$ upregulation is also considerably less than that of the primed superoxide response.

5.4 Effects of PAF, $TNF\alpha$ and LPS on Basal and fMLP-stimulated GTP Hydrolysis in Human Neutrophil Membranes

To confirm that the enhanced expression of membrane $G_{i\alpha 2}$ detected above is active in coupling receptor to effector, basal and stimulated GTP hydrolysis were measured in identically prepared membranes from unprimed and primed cells. Since insufficient membrane protein was available from the previous experiments to perform this analysis, incubation conditions resulting in maximal membrane G-protein expression (PAF 100 nM for 10 min, $TNF\alpha$ 200 U/ml for 30 min and LPS 100 ng/ml for 120 min) were used to prepare fresh membrane samples. As can be seen from Figure 5.5, while all three priming agents enhanced both basal and fMLP-stimulated GTP hydrolysis, the greatest effect on the fMLP-stimulated response was seen with PAF, while LPS and $TNF\alpha$ only induced a very modest increase in membrane GTP hydrolytic activity. In addition, the enhancement of GTP hydrolysis observed following incubation with priming agents was of lesser magnitude than the enhancement of $G_{i\alpha 2}$ expression (compare Figures 5.1-5.4 with Figure 5.5). This suggests that either not all the G-protein recruited to the plasma membrane was fully active or that $G_{i\alpha 2}$ does not represent the major component of neutrophil plasma membrane GTP-ase activity.

Figure 5.5

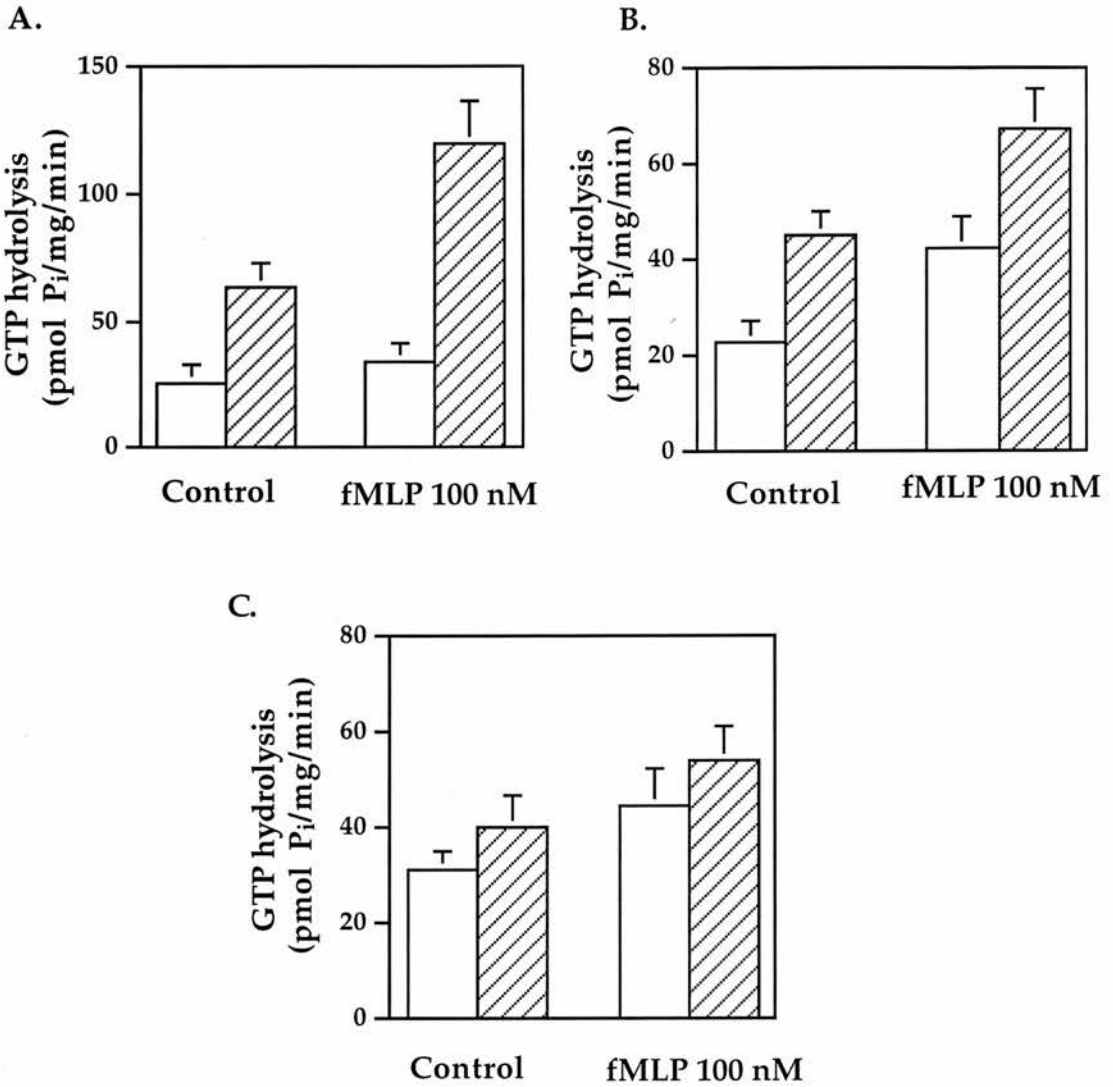


Figure 5.5 Effects of PAF, TNF α and LPS on Basal and fMLP-stimulated GTP Hydrolysis in Human Neutrophil Membranes

Human neutrophils incubated with buffer (open bars) or priming agent (hatched bars) were disrupted by nitrogen cavitation; the plasma membrane fraction was separated by Percoll gradient centrifugation, and hydrolysis of [γ - 32 P]GTP was determined (by quantitating the release of free [32 P] P_i , see 2.6.3). (A) PAF 100 nM (10 min), (B) TNF α 200 U/ml (30 min) and (C) LPS 100 ng/ml (120 min); results are expressed as mean \pm sem, n=4 from 2 separate experiments.

5.5 Discussion

The results presented in this chapter have demonstrated that the three priming agents studied (PAF, $\text{TNF}\alpha$ and LPS) all enhanced the detection in isolated neutrophil plasma membranes of $\text{G}_{i\alpha 2}$, the most abundant neutrophil G-protein. This enhancement occurred rapidly (within 2 min) in response to PAF but was delayed in onset (120 min) when LPS was used, whilst the effects of $\text{TNF}\alpha$ were near-maximal by 10 min. The time-course for $\text{G}_{i\alpha 2}$ upregulation was thus shown to reflect the times required for each agent to prime neutrophils in vitro (Chapter 3, Figure 3.6), suggesting initially that enhanced signal transduction via G-proteins coupled to the fMLP receptor could contribute to priming in a mechanistic way. Indeed, this has been the precise conclusion of three previous studies examining the effects of single priming agents (LPS, GM-CSF and $\text{TNF}\alpha$) on $\text{G}_{i\alpha 2}$ distribution and function (Yasui et al., 1992, Durstin et al., 1993 and Klein et al., 1995). The data shown in Fig 5.5 also confirmed that membranes from primed neutrophil possess increased GTP-ase activity and hence that at least some of the recruited $\text{G}_{i\alpha 2}$ was functional.

The use of nitrogen cavitation to obtain purified plasma membranes has previously been shown to minimise exposure to lysosomal hydrolytic enzymes and to yield membrane vesicles free of other subcellular organelles (Klempner et al., 1980, Borregaard et al., 1983). However, even though this technique represents an improvement on previous methods employing mechanical shear forces to disrupt cells, it clearly has the potential to disrupt significantly subcellular organisation. In intact cells, some sets of receptors, G-proteins and effectors may be compartmentalised into microdomains and not have access to other sets; making membrane preparations from erythrocytes increases the mobility of membrane proteins by more than an

order of magnitude (Beth et al., 1986). Thus caution must be applied in extrapolating results obtained from isolated membrane preparations to intact, functioning cells.

Potential sources of the additional $G_{i\alpha 2}$ recruited to the neutrophil plasma membrane include the cytosol, specific granule stores and de novo protein synthesis. Yasui et al. (1992) demonstrated that LPS in the presence of serum increased the level of $G_{i\alpha 2}$ present in the membrane fraction but not in whole cell preparations of human neutrophils, and Durstin et al. (1993) made a similar observation in GM-CSF-treated neutrophils, G-protein recruitment in the latter study being unaffected by the protein synthesis inhibitor cycloheximide. These data, together with the rapidity of the response to PAF and $TNF\alpha$, suggest that redistribution of $G_{i\alpha 2}$, rather than synthesis of additional protein, accounts for the increased presence of pre-formed $G_{i\alpha 2}$ within the plasma membrane fraction.

Intracellular granule membranes serve as a reservoir of proteins whose mobilisation to the cell surface during limited exocytosis plays an important role in leukocyte chemotaxis and activation, and the specific granule fraction has been identified as an important intracellular reserve of fMLP receptors (Fletcher and Gallin, 1983), adhesion molecules (Bainton et al., 1987) and as a translocatable pool of cytochrome b_{558} (Calafat et al., 1993). Rotrosen et al. (1988) demonstrated by Western blotting of fractionated human neutrophils that $G_{i\alpha}$ was present in the plasma membrane and specific granule-enriched fraction; approximately 60% of total pertussis toxin substrate localised to the plasma membrane, 35% to the specific granule-enriched fraction and 5% to the cytosol. These figures tally well with those of Durstin et al. (1993). Both authors additionally noted that treatment with agonist (fMLP or GM-CSF) resulted in increased plasma membrane-associated G-protein concomitant with an equivalent decrease in the granule-associated G-protein. It should

be noted, however, that both studies employed human neutrophils isolated from peripheral blood using Ficoll-Hypaque gradients and hypotonic lysis, which may in itself induce priming (Haslett et al., 1985) and hence affect cellular G-protein distribution. Finally, the data of Rotrosen et al. (1988) suggested that the intracellular reserve of fMLP receptors might be functionally coupled to $G_{i\alpha}$ before translocation to the plasma membrane. The structural and functional upregulation of $G_{i\alpha 2}$ could therefore represent a selective degranulation response to the priming agents. The time-course of membrane-associated $G_{i\alpha 2}$ upregulation correlated well with increased detection of surface CD11b (see Figure 4.5), an event also felt to reflect degranulation, although enhanced levels of plasma membrane $G_{i\alpha 2}$ were seen at slightly lower agonist concentrations than those inducing CD11b translocation (Chapter 4, Figures 4.2-4.4 versus Figure 8.4). The small fraction of cytosolic $G_{i\alpha 2}$ is a further potential source of translocatable protein; reversible palmitoylation of an N-terminal cysteine residue is thought to modulate G_{α} membrane affinity, and mutation of these palmitoylation sites may result in an alteration in the distribution of the proteins between the particulate fraction and the supernatant (reviewed by Milligan et al., 1995). However, the influence of palmitoylation on neutrophil G-proteins is as yet unknown.

The diversity of G-protein subunits imparts great versatility to G-protein-mediated signalling (Simon et al., 1991). In neutrophils, the G_i family is represented by only two of its members, $G_{i\alpha 2}$ and $G_{i\alpha 3}$, the main pertussis toxin substrates in these cells (Falloon et al., 1986, Murphy et al., 1987, Uhing et al., 1987), whilst G_o is absent (Murphy et al., 1987). Activation of G_s , for example by stimulation of β -adrenoceptors, results in inhibition of O_2^- generation (Mueller et al., 1991). A member of the G_q family, $G_{\alpha 16}$, is specifically expressed in haemopoietic cells (Amatruda et al., 1991); however,

levels of $G\alpha_{16}$ diminish with neutrophilic differentiation; furthermore $G\alpha_{16}$ is not a pertussis toxin substrate and has not been shown to functionally couple to the fMLP receptor, hence it is unlikely that this G-protein plays an important role in the priming of O_2^- generation in the mature neutrophil.

Functional coupling of the fMLP receptor to both $G_{i\alpha 2}$ and $G_{i\alpha 3}$ was demonstrated in myeloid-differentiated HL-60 cells by Gierschik et al. (1989), and Klein et al. (1995) demonstrated that fMLP receptors were functionally coupled to the increased $G_{i\alpha}$ detected in $TNF\alpha$ -treated neutrophil membranes, implicating this G-protein family in the signal transduction process of the primed respiratory burst. Although increased plasma membrane $G_{i\alpha 2}$ expression was seen in response to three diverse priming agents, and the time-course of this event correlated with the priming effect of these agents, the maximal upregulation of $G_{i\alpha 2}$ expression observed in the experiments presented above was less than threefold compared to the tenfold or greater enhancement of fMLP-stimulated O_2^- release seen in similarly primed cells (Chapter 3, Figures. 3.3-3.5). In addition, the much greater effect of PAF observed on $G_{i\alpha 2}$ expression was not mirrored by a similarly greater augmentation of O_2^- priming. These findings both suggest that mechanisms other than modulation of $G_{i\alpha 2}$ distribution are involved in the priming response.

In summary, PAF, $TNF\alpha$ and LPS increase the level of membrane-associated $G_{i\alpha 2}$ with a time-course which correlates closely with priming of fMLP-stimulated O_2^- generation but not with either the magnitude or the efficacy rank order for the individual priming agents.

Chapter 6: The Role of Inositol 1,4,5-Trisphosphate in Neutrophil Priming

6.1 Introduction

The enhanced turnover of inositol lipids in response to receptor activation is one of the major mechanisms utilised by cells for transmembrane signalling. The initial event in this pathway is the phosphodiesteric cleavage of the integral plasma membrane phospholipid phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P₂) by phosphoinositide-specific phospholipase C (PIC) to yield the second messengers inositol 1,4,5-trisphosphate (Ins(1,4,5)P₃) and diacylglycerol (DAG). The physiological significance of phosphoinositide hydrolysis was not fully appreciated until Michell (1975) predicted a link between this event and calcium signalling; eight years later, Streb et al. (1983) demonstrated Ins(1,4,5)P₃-induced Ca²⁺ release from intracellular Ca²⁺ stores, subsequently shown to be mediated by an Ins(1,4,5)P₃-gated Ca²⁺ channel (reviewed by Berridge, 1993). DAG was shown to activate protein kinase C (reviewed by Nishizuka, 1984).

Changes in [Ca²⁺]_i homeostasis have been widely implicated in the priming process. In particular, Forehand et al. (1989) demonstrated that priming with LPS caused an increase in basal [Ca²⁺]_i and augmented the size and duration of the fMLP-induced [Ca²⁺]_i transient. Further support for a role for [Ca²⁺]_i in neutrophil priming was provided by Finkel et al. (1987) who correlated cytosolic Ca²⁺ (manipulated by means of the calcium ionophore ionomycin) with priming for enhanced fMLP-triggered O₂⁻ release. Yee and Christou (1993) studied this phenomenon by flow cytometry in LPS-primed, fluo-3-AM-loaded neutrophils, demonstrating that in the primed population a higher percentage of individual cells responded to low concentrations of

fMLP (≤ 10 nM) with calcium transients, which were also prolonged with respect to those seen in unprimed cells. The importance of calcium transients in the activation of the NADPH oxidase was emphasised by the work of Hallett et al. (1990), who demonstrated a tight coupling in individual neutrophils between an elevation of $[Ca^{2+}]_i$ above a threshold of 250 nM and O_2^- production. However, this may not represent the sole signalling route leading to priming, since in another report, $TNF\alpha$ was found neither to elevate basal $[Ca^{2+}]_i$ nor to affect fMLP-stimulated Ca^{2+} transients (Yuo et al., 1989).

In neutrophils the major route of PIC (the β_2 isoform, see Chapter 1.8.3) activation is via an interaction with the dissociated $\beta\gamma$ -subunit of a heterotrimeric G-protein, released following the binding of ligand to the coupled transmembrane receptor (Camps et al., 1992, Katz et al., 1992). Both LPS and PAF have been shown to stimulate $PtdIns(4,5)P_2$ hydrolysis directly in macrophages (Prpic et al., 1987, Huang et al., 1988), maximal $Ins(1,4,5)P_3$ levels being achieved at 10-15 s with both agonists, followed by a slow decline (5-10 min). PAF also elevated $Ins(1,4,5)P_3$ and Ca^{2+} in neutrophils (Molski et al., 1988, Grimminger et al., 1991), but few studies have addressed the possibility that priming agents may enhance $Ins(1,4,5)P_3$ responses to secretagogue agonists. Such a possibility would be supported by the aforementioned studies demonstrating that certain priming agents enhanced fMLP-mediated Ca^{2+} release and by the observation that GM-CSF enhanced $PtdIns$ synthase activity in neutrophils (Macphee, 1992). A precedent exists for the potentiation of PIC activation; Marin et al. (1991) demonstrated that somatostatin, which is ineffective alone, enhanced the production of inositol phosphates and prolonged the elevation of $[Ca^{2+}]_i$ in cultured striatal astrocytes stimulated with α_1 -adrenergic agonists.

Since we have demonstrated that the priming agents LPS, $TNF\alpha$ and PAF

caused translocation of $G_{i\alpha 2}$ to the neutrophil plasma membrane in a time- and concentration-dependent fashion closely correlated with their priming properties (Chapter 5), and since Ca^{2+} has been implicated in neutrophil activation (Dewald et al., 1988), we chose to study the potential role of enhanced $Ins(1,4,5)P_3$ formation in neutrophil priming. In particular, we wished to elucidate whether enhanced fMLP-induced $Ins(1,4,5)P_3$ generation could underlie primed O_2^- release, as the fMLP receptor is coupled (at least in HL-60 cells) to PIC via $G_{i\alpha 2}$ and $G_{i\alpha 3}$, (Uhing et al., 1987, Gierschik et al., 1989).

6.2 Efficacy of Priming of Human Neutrophils at High Cell Density (7×10^6 /225 μ l)

The use of the Ins(1,4,5) P_3 mass assay necessitated the use of approximately 7×10^6 neutrophils per assay point; attempts to analyse neutrophil Ins(1,4,5) P_3 mass under the conditions previously used to establish priming of the O_2^- (i.e. 1×10^6 cells per assay point) by freeze-drying the neutralised extracted lipids and redissolving in a smaller volume proved unsuccessful, generating inconsistent and poorly reproducible Ins(1,4,5) P_3 values even from duplicate samples (not shown). We therefore confirmed that priming was effective under the conditions to be used in this assay (5-10 fold increase in the fMLP-triggered O_2^- response, Figure 6.1).

6.3 The Effects of LPS, TNF α and PAF on Peak fMLP-stimulated Ins(1,4,5) P_3 Accumulation in Human Neutrophils.

It was established in preliminary experiments (not shown, but see subsequent time-course data, Figures 6.5-6.8) that maximal Ins(1,4,5) P_3 accumulation following stimulation with fMLP 100 nM was achieved at 10 s; sub-maximal responses with lower concentrations of fMLP (1 and 10 nM) demonstrated similar kinetics. We therefore determined Ins(1,4,5) P_3 mass in control and primed neutrophils at 10 s after exposure to 1-100 nM fMLP; priming was achieved using the conditions previously demonstrated to be effective (Figure 6.1). The results of these experiments are shown in Figures 6.2-6.4. In unprimed cells, formylated peptide elevated the Ins(1,4,5) P_3 mass detected at 10 s in a concentration-dependent fashion, with an $EC_{50} \approx 10$ nM and a maximal response (2.3-3 fold increase) to 100 nM fMLP. Increasing the concentration of fMLP did not further augment Ins(1,4,5) P_3 accumulation (not shown). Incubation of neutrophils with 100 nM PAF alone for 5 min resulted in a significant elevation of Ins(1,4,5) P_3 mass above that seen in

Figure 6.1

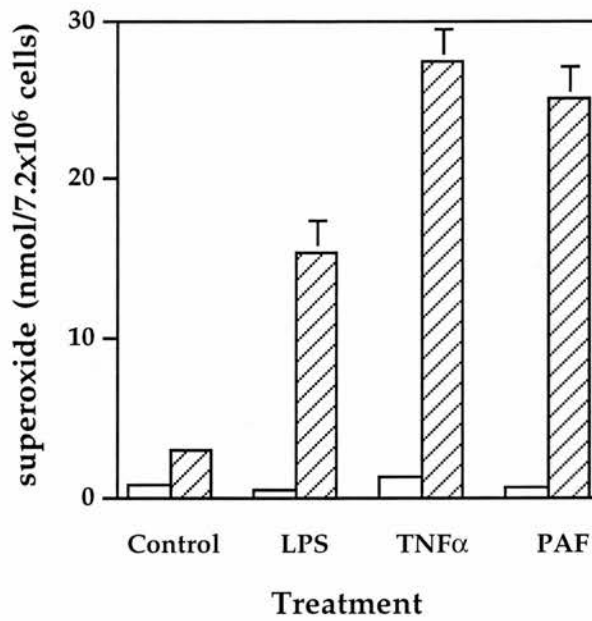


Figure 6.1 Priming of Human Neutrophil O_2^- Release at High Cell Density Human neutrophils (7.2×10^6 in 225 μ l PBS with calcium and magnesium) were incubated at 37°C with or without priming agents (LPS 100 ng/ml, 120 min; TNF α 200 U/ml, 30 min; or PAF 100 nM, 10 min) prior to the addition of cytochrome C (1.2 mg/ml final concentration) and of fMLP (final concentration 100 nM, hatched bars) or vehicle (open bars) in a final volume of 1 ml. SOD (375 U) was included in one of each set of quadruplicate incubations and O_2^- release determined by the superoxide dismutase-inhibitable reduction of cytochrome C as described in (2.3). Data represent mean \pm sem of 2 separate experiments, each performed in triplicate.

Figure 6.2

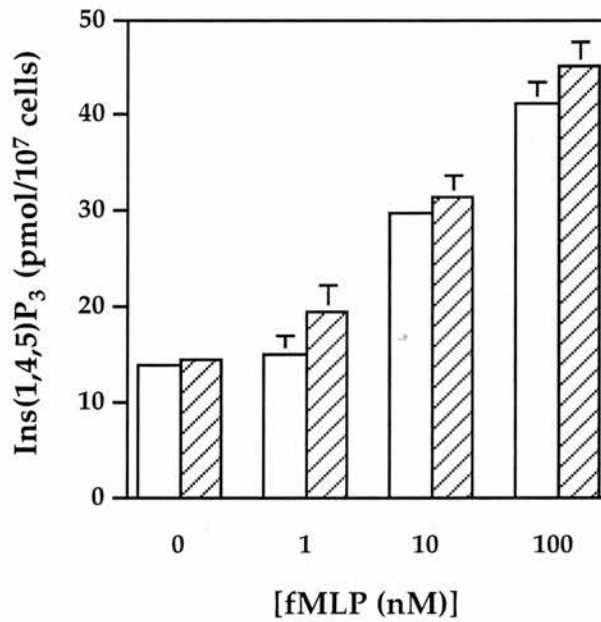


Figure 6.2 Effects of LPS Priming on Human Neutrophil Ins(1,4,5)P₃ Mass in Resting and fMLP-stimulated Cells

Neutrophils (7.2×10^6 in 225 μ l PBS with calcium and magnesium) were incubated with (hatched bars) or without (open bars) LPS 100 ng/ml (added in 25 μ l for 120 min at 37°C) prior to stimulation with fMLP 1-100 nM (50 μ l) or vehicle; the reactions were stopped at 10 s by the addition of 60 μ l ice cold 3 M TCA with vortexing. The samples were allowed to extract at 4°C for 30 min and were then partitioned with freon/octylamine (1:1, v/v) as described in (2.7.2). The upper phases (150 μ l) were neutralised (60 mM NaHCO₃, 40 μ l), and the Ins(1,4,5)P₃ mass of the neutralised extracts determined exactly as described (2.7.3). Results represent the mean \pm sem of 5 separate experiments each performed in triplicate.

Figure 6.3

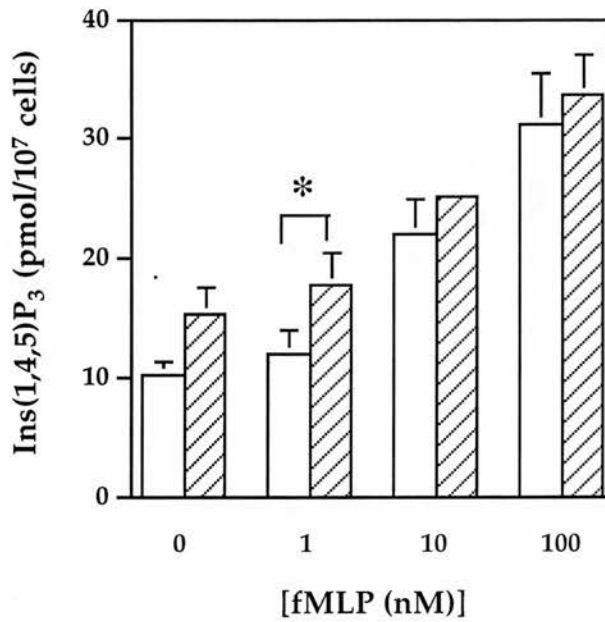


Figure 6.3 Effects of TNF α Priming on Human Neutrophil Ins(1,4,5)P₃ Mass in Resting and fMLP-stimulated Cells

Neutrophils (7.2×10^6 in 225 μ l PBS with calcium and magnesium) were incubated with (hatched bars) or without (open bars) TNF α 200 U/ml (added in 25 μ l) for 30 min at 37°C, prior to stimulation with fMLP 1-100 nM (50 μ l) or vehicle; the reactions were stopped at 10 s by the addition of 60 μ l ice cold 3 M TCA with vortexing. The samples were allowed to extract at 4°C for 30 min and were then partitioned with freon/octylamine (1:1, v/v) as described in (2.7.2). The upper phases (150 μ l) were neutralised (60 mM NaHCO₃, 40 μ l), and the Ins(1,4,5)P₃ mass of the neutralised extracts determined exactly as described (2.7.3). Results represent the mean \pm sem of 5 separate experiments each performed in triplicate. * denotes $p < 0.05$ (Student's paired t-test).

Figure 6.4

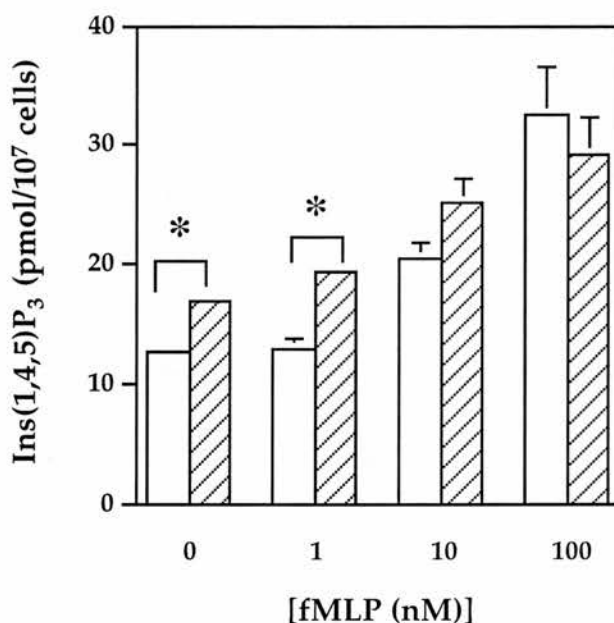


Figure 6.4 Effects of PAF Priming on Human Neutrophil Ins(1,4,5)P₃ Mass in Resting and fMLP-stimulated Cells

Neutrophils (7.2×10^6 in 225 μ l PBS with calcium and magnesium) were incubated with (hatched bars) or without (open bars) PAF 100 nM (added in 25 μ l) for 10 min at 37°C, prior to stimulation with fMLP 1-100 nM (50 μ l) or vehicle; the reactions were stopped at 10 s by the addition of 60 μ l ice cold 3 M TCA with vortexing. The samples were allowed to extract at 4°C for 30 min and were then partitioned with freon/octylamine (1:1. v/v) as described in (2.7.2). The upper phases (150 μ l) were neutralised (60 mM NaHCO₃, 40 μ l), and the Ins(1,4,5)P₃ mass of the neutralised extracts determined exactly as described (2.7.3). Results represent the mean \pm sem of 3 separate experiments each performed in triplicate. * denotes $p < 0.05$ (Student's paired t-test).

control (unprimed) cells (Figure 6.4); changes in resting Ins(1,4,5)P₃ mass following priming with LPS 100 ng/ml (120 min) or with TNF α 200 U/ml (30 min) did not reach statistical significance (Figures 6.2 and 6.3). Significant differences in fMLP-stimulated Ins(1,4,5)P₃ accumulation were detected between unprimed cells and cells primed with PAF and TNF α when 1 nM fMLP was used as the stimulus; however the small augmentation of fMLP-induced Ins(1,4,5)P₃ accumulation observed at these low fMLP concentrations was not significantly different from that seen with priming agent alone (Figures 6.3 and 6.4). Priming did not affect Ins(1,4,5)P₃ accumulation in response to fMLP at 10 s to higher concentrations of agonist (Figures 6.2-6.4).

Since priming appeared to have little effect on Ins(1,4,5)P₃ responses to fMLP measured at 10 s, we decided to examine the kinetics of Ins(1,4,5)P₃ accumulation in greater detail, to exclude an effect on the rapidity of onset or duration of phosphoinositide hydrolysis or any independent influence of priming agents on Ins(1,4,5)P₃ metabolism. PAF (a known Ca²⁺-mobilising agent) and TNF α (which does not mobilise Ca²⁺) were selected for use in the following time-course experiments.

6.4 The Effects of TNF α on the Time-course of fMLP-stimulated Ins(1,4,5)P₃ Accumulation

The effects of TNF α on fMLP-stimulated Ins(1,4,5)P₃ mass were determined at time points ranging from 0-10 min, using both 100 nM fMLP (the concentration used to illustrate priming of O₂⁻ generation) and 1 nM fMLP (at which concentration a significant difference was detected between primed and unprimed cells, Figure 6.3) as the stimulating agent. The results depicted in Figures 6.5 and 6.6 illustrate that TNF α exerts minimal effects on the time-course of Ins(1,4,5)P₃ accumulation. No significant differences in

Figure 6.5

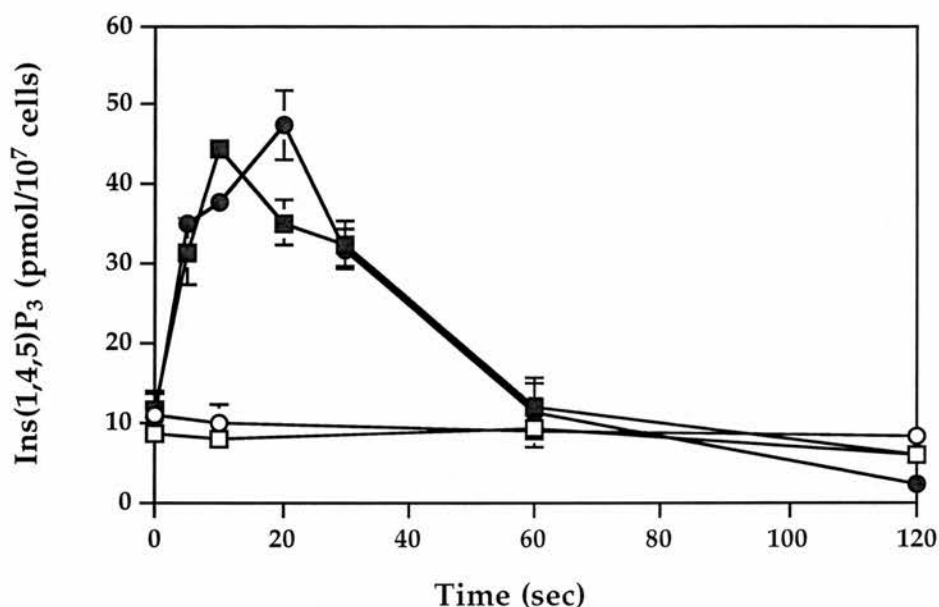


Figure 6.5 Effects of TNF α Priming on 100 nM fMLP-stimulated Ins(1,4,5)P₃ Accumulation in Human Neutrophils.

Neutrophils (7.2×10^6 in 225 μ l PBS with calcium and magnesium) were incubated with (circles) or without (squares) TNF α 200 U/ml for 30 min at 37°C, prior to stimulation with fMLP 100 nM (closed symbols) or vehicle (open symbols); the reactions were stopped at the appropriate times (0-120 s) by the addition of 60 μ l ice cold 3 M TCA. The samples were allowed to extract at 4°C for 30 min and were then partitioned with freon/octylamine (1:1, v/v) as described in (2.7.2). The upper phases (150 μ l) were neutralised with 60 mM NaHCO₃, and the Ins(1,4,5)P₃ mass of the neutralised extracts determined exactly as described (2.7.3). Results represent the mean \pm sem of 3 separate experiments each performed in duplicate.

Figure 6.6

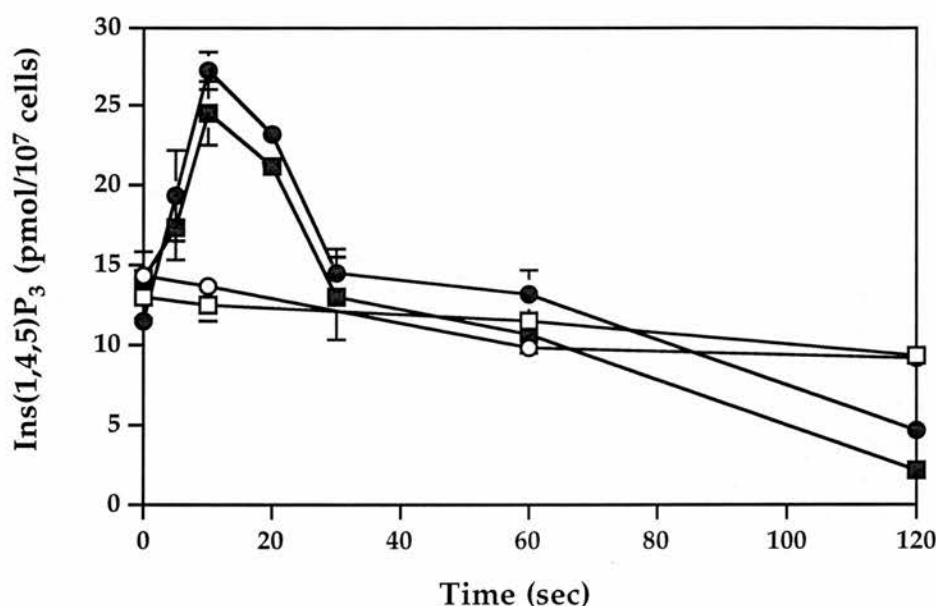


Figure 6.6 Effects of $\text{TNF}\alpha$ Priming on 1 nM fMLP-stimulated $\text{Ins}(1,4,5)\text{P}_3$ Accumulation in Human Neutrophils.

Neutrophils (7.2×10^6 in 225 μl PBS with calcium and magnesium) were incubated with (circles) or without (squares) $\text{TNF}\alpha$ 200 U/ml for 30 min at 37°C , prior to stimulation with fMLP 1 nM (closed symbols) or vehicle (open symbols); the reactions were stopped at the appropriate times (0-120 s) by the addition of 60 μl ice cold 3 M TCA. The samples were allowed to extract at 4°C for 30 min and were then partitioned with freon/octylamine (1:1, v/v) as described in (2.7.2). The upper phases (150 μl) were neutralised with 60 mM NaHCO_3 , and the $\text{Ins}(1,4,5)\text{P}_3$ mass of the neutralised extracts determined exactly as described (2.7.3). Results represent the mean \pm sem of 3 separate experiments each performed in duplicate.

Table 6.1

	5 min post fMLP		10 min post fMLP	
Stimulus	Unprimed	Primed	Unprimed	Primed
fMLP 1 nM	10.7±0.2	10.7±0.3	12.8±0.8	13.0±2.3
fMLP 100 nM	11.81±0.9	13.0±0.4	12.9±0.4	13.8±1.9

Table 6.1 Effects of TNF α Priming on fMLP-stimulated Ins(1,4,5)P₃ Accumulation at 5 and 10 min

Neutrophils (7.2x10⁶ in 225 μ l PBS with calcium and magnesium) were incubated with or without TNF α 200 U/ml (added in 25 μ l) for 30 min at 37°C, prior to stimulation with 1 nM or 100 nM fMLP (50 μ l) or vehicle; the reaction was stopped at 5 or 10 min by the addition of 60 μ l ice cold 3 M TCA with vortexing. The samples were allowed to extract at 4°C for 30 min and were then partitioned with freon octylamine as described in (2.7.2). The upper phases (150 μ l) were neutralised (60 mM NaHCO₃, 40 μ l), and the Ins(1,4,5)P₃ mass of the neutralised lipid extracts was determined exactly as described (2.7.3). Results (pmol/10⁷ cells) represent the mean \pm sem of 2 separate experiments each performed in duplicate.

Ins(1,4,5)P₃ accumulation in response to 1 nM or 100 nM fMLP were seen between 0-120 s following fMLP addition, although the response to 100 nM fMLP was slightly prolonged; the marginal enhancement of the 10 s peak response to 1 nM fMLP did not reach statistical significance in this series of experiments. In separate experiments, Ins(1,4,5)P₃ mass was determined 5 and 10 min following stimulation; again, no significant differences between primed and unprimed cells were seen with either concentration of fMLP (Table 6.1).

6.5 Effects of PAF on the Time-course of fMLP-stimulated Ins(1,4,5)P₃ Accumulation

From Figure 6.7 it can be seen that the time-courses of Ins(1,4,5)P₃ accumulation in unprimed and PAF-primed cells were superimposable when 100 nM fMLP was applied. The data for 1 nM fMLP confirm the previously detected enhancement of Ins(1,4,5)P₃ accumulation in PAF-primed cells at 10 s, and values at 5 and 30 s are also slightly elevated (Figure 6.8); however, a higher baseline (unstimulated) level of Ins(1,4,5)P₃ was also detected in primed neutrophils compared to the unprimed controls. The increase in basal Ins(1,4,5)P₃ in PAF-primed cells is similar in magnitude to the augmentation of fMLP-stimulated Ins(1,4,5)P₃ detection, and is probably sufficient alone to explain the post-fMLP enhancement.

Figure 6.7

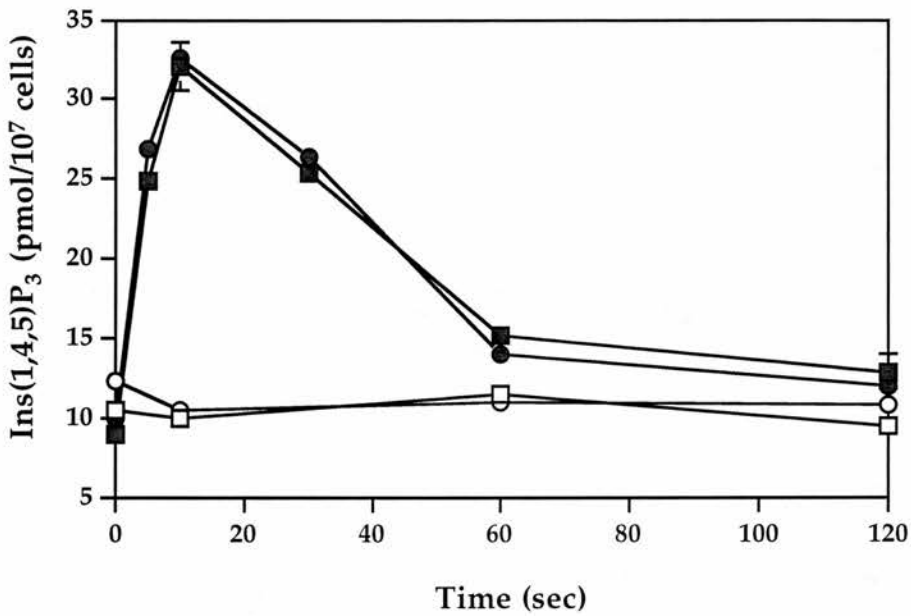


Figure 6.7 Effects of PAF Priming on 100 nM fMLP-stimulated Ins(1,4,5)P₃ Accumulation in Human Neutrophils.

Neutrophils (7.2×10^6 in 225 μ l PBS with calcium and magnesium) were incubated with (circles) or without (squares) PAF 100 nM for 10 min at 37°C, prior to stimulation with fMLP 100 nM (closed symbols) or vehicle (open symbols); the reactions were stopped at the appropriate times (0-120 s) by the addition of 60 μ l ice cold 3 M TCA. The samples were allowed to extract at 4°C for 30 min and were then partitioned with freon/octylamine (1:1,v/v) as described in (2.7.2). The upper phases (150 μ l) were neutralised with 60 mM NaHCO₃, and the Ins(1,4,5)P₃ mass of the neutralised extracts determined exactly as described (2.7.3). Results represent the mean \pm sem for n=2 duplicate determinations.

Figure 6.8

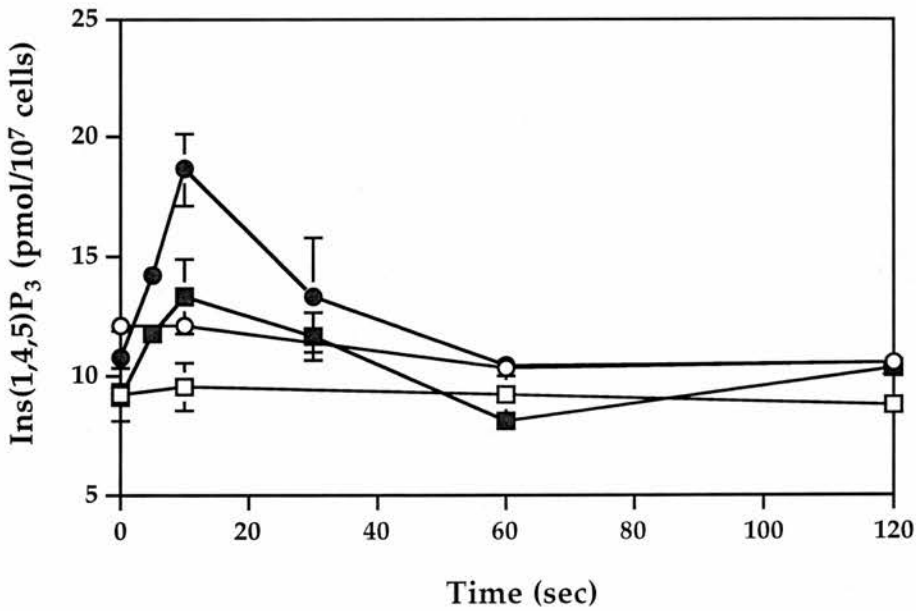


Figure 6.8 Effects of PAF Priming on 1 nM fMLP-stimulated Ins(1,4,5)P₃ Accumulation in Human Neutrophils.

Neutrophils (7.2×10^6 in 225 μ l PBS with calcium and magnesium) were incubated with (circles) or without (squares) PAF 100 nM for 10 min at 37°C, prior to stimulation with fMLP 1 nM (closed symbols) or vehicle (open symbols); the reactions were stopped at the appropriate times (0-120 s) by the addition of 60 μ l ice cold 3 M TCA. The samples were allowed to extract at 4°C for 30 min and were then partitioned with freon/octylamine (1:1, v/v) as described in (2.7.2). The upper phases (150 μ l) were neutralised with 60 mM NaHCO₃, and the Ins(1,4,5)P₃ mass of the neutralised extracts determined exactly as described (2.7.3). Results represent the mean \pm sem for n=2 duplicate determinations.

6.6 Discussion

We have demonstrated that fMLP at a concentration of 100 nM induced maximal Ins(1,4,5)P₃ accumulation that could not be augmented by preincubation under optimal priming conditions with LPS, TNF α or PAF; under identical conditions fMLP alone caused minimal activation of the NADPH oxidase, but O₂⁻ generation was enhanced 5-10 fold following exposure to a priming agent. These data argue strongly against a role for augmentation of Ins(1,4,5)P₃-mediated Ca²⁺ release in neutrophil priming. While a statistically significant increase in Ins(1,4,5)P₃ mass at 10 s was detected in PAF-primed versus unprimed neutrophils stimulated with 1 nM fMLP, the results were no more than additive; indeed, the values obtained did not differ significantly from those obtained for cells treated with PAF alone (Fig 6.4). Neutrophils primed with PAF (100 nM, 10 min) exhibited significantly higher unstimulated Ins(1,4,5)P₃ mass than controls, presumably reflecting persisting PAF-induced phosphoinositide hydrolysis. Priming with TNF α also resulted in a slight elevation of resting Ins(1,4,5)P₃ mass (not reaching statistical significance) and of the mass detected following 10 s stimulation with 1 nM fMLP ($p < 0.05$, Fig 6.3); these results are surprising in view of the generally accepted dictum that TNF α does not mobilise Ca²⁺ in human neutrophils. However, TNF α -induced [Ca²⁺]_i oscillations have been reported in adherent human neutrophils (Schumann et al., 1993). Whilst the neutrophils in our experiments were kept in suspension, the higher cell density (7.2x10⁶/ml) may have promoted cell-cell interactions, thereby simulating some of the conditions pertaining to the adherent state (although cells incubated at this density did not produce O₂⁻ in response to TNF α alone (Figure 6.1), in contrast to the studies of Nathan et al., 1987 and 1989a, using adherent neutrophils). A precedent for modified

phosphoinositide metabolism in adherent cells exists; McNamee et al. (1993) demonstrated that adhesion of a fibroblast cell line (CH3 10Y1/2) to fibronectin increased the synthesis of $\text{PtdIns}(4,5)\text{P}_2$ and enhanced PDGF-stimulated inositol phosphate release.

The use of a mass assay to measure $\text{Ins}(1,4,5)\text{P}_3$ obviates the need for a prolonged (2 h or greater) incubation of neutrophils with $[^3\text{H}]\text{inositol}$, with possible adverse functional consequences; such labelling artefact may underlie the discrepancies between the reports of MacPhee (1992) and of Corey and Rosoff (1989), the former demonstrating enhanced, and the latter unchanged accumulation of $[^3\text{H}]\text{Ins}(1,4,5)\text{P}_3$ in neutrophils primed with GM-CSF and stimulated with fMLP. The use of plasma/Percoll-prepared cells in combination with the mass assay technique allowed us to contrast the intracellular signals of truly unprimed cells with deliberately primed cells; the remarkably similar $\text{Ins}(1,4,5)\text{P}_3$ profiles contrast sharply with the previously demonstrated functional differences. In many experiments, following stimulation with fMLP a fall of $\text{Ins}(1,4,5)\text{P}_3$ to levels below baseline was seen (e.g. Figure 6.6); this undershoot phenomenon has been previously reported in bovine tracheal smooth muscle cells (Chilvers et al., 1989) and has been attributed to agonist-stimulated $\text{Ins}(1,4,5)\text{P}_3$ metabolism (Chilvers et al., 1991).

How can the results presented in this chapter be reconciled with those of Forehand et al. (1989), who demonstrated elevated baseline $[\text{Ca}^{2+}]_i$ and an enhanced and prolonged Ca^{2+} spike in LPS-primed cells? Ca^{2+} transients were measured by Forehand utilising the Ca^{2+} -sensitive indicator dye, fura-2/AM. Although this dye is superior to pre-existing reagents (Grynkiewicz et al., 1985), its use still presents problems (discussed by Marks et al., 1990, and Schumann et al., 1993) including buffering of $[\text{Ca}^{2+}]_i$ by the dye, and the generation of cellular autofluorescence due to excitation of the dye at UV

wavelengths. Different Ca^{2+} indicators may give rise to disparate data even in the same cell type (Williamson and Monck, 1989). We have also found that standard loading of neutrophils with 2 μM fura-2/AM for 30 min leads to a slight degree of priming, mainly attributable to the DMSO in which the dye is dissolved (nmol O_2^- generated per 10^6 cells in response to 100 nM fMLP: control (PBS) 3.4 ± 1.2 , fura-2/AM 5.9 ± 1.6 , and 0.1% DMSO (vehicle) 5.2 ± 0.9). While these limitations of Ca^{2+} -indicators are generally accepted, they are unlikely to account for differences between unprimed/primed cells loaded together or in parallel. Priming agents could theoretically alter the uptake and cellular distribution of fura-2/AM or its cleavage to fura-2, or induce changes that quench fura-2-generated fluorescence, but these issues have not as yet been addressed experimentally.

Studies of $[\text{Ca}^{2+}]_i$ at a single cell level indicate that priming probably results in the recruitment of a proportion of cells into a more highly responsive pool, with heterogeneous calcium responses observed following LPS priming (Yee and Christou, 1993) and in response to fMLP, C5a and IL-8 (Elsner et al., 1992); additionally, Elbim et al. (1994) demonstrated recruitment of cells into an fMLP-responsive H_2O_2 -producing neutrophil pool subsequent to priming with $\text{TNF}\alpha$, GM-CSF, and, to a lesser extent, IL-8. Heterogeneity may also exist at the level of the $\text{Ins}(1,4,5)\text{P}_3$ receptor. Although a family of at least three receptors has been characterised, Sugiyama et al. (1994) found that rat neutrophils expressed predominantly the type 2 receptor, which exhibited a significantly higher affinity for $\text{Ins}(1,4,5)\text{P}_3$ than the originally described cerebellar type 1 receptor despite significant sequence homology. The density of $\text{Ins}(1,4,5)\text{P}_3$ -sensitive Ca^{2+} channels within intracellular Ca^{2+} stores was found to be non-uniform in vascular smooth muscle cells by Hirose and Iino (1994), although these data have not yet been extended to other cell types. Additionally, the affinity of

the Ins(1,4,5)P₃ receptor for its ligand may be modulated, by the oxidative state of critical receptor sulfhydryl groups (Kaplin et al., 1994) or by receptor phosphorylation (discussed by Fisher, 1995). Small, localised changes in Ins(1,4,5)P₃ concentration (possibly at a subplasmalemmal location) in only a proportion of cells could therefore be important in signalling the primed state, with currently available assays being insufficiently sensitive to detect such fluctuations.

A further complication is that Ca²⁺ flux may be regulated by mechanisms distinct from PtdIns(4,5)P₂ hydrolysis. Rigley et al. recently (1995) demonstrated a role in Ca²⁺ signalling for the non-receptor tyrosine kinase p59^{fyn(T)} in Jurkat T cells; decreased expression of p59^{fyn(T)} (produced by transfection with anti-sense cDNA) led to a reduced Ca²⁺ influx in response to cross-linking of the T-cell receptor, whilst Ins(1,4,5)P₃ production was not attenuated. Sphingosine (an endogenous sphingolipid) was found to elevate human neutrophil [Ca²⁺]_i with no effect on basal or fMLP-stimulated Ins(1,4,5)P₃ generation (Wong and Kwan-Yeung, 1993). Finally, Jurkat T cells were reported to possess ryanodine receptors (Guse et al., 1993; discussed in Murphy et al., 1995), raising the possibility of the involvement of cyclic adenosine diphosphate-ribose (cADPR; Galione et al., 1993), the presumptive physiological regulator of Ins(1,4,5)P₃-insensitive Ca²⁺ release via ryanodine receptor-like channels. Hence it is still possible that Ca²⁺ transients may have a role in neutrophil priming, but any differences would appear to be independent of Ins(1,4,5)P₃ accumulation.

In summary, priming did not augment or prolong Ins(1,4,5)P₃ accumulation stimulated by 100 nM fMLP, and the minor augmentation seen when 1 nM fMLP was used as a stimulus was probably an additive effect. While this makes a major role for Ins(1,4,5)P₃ in neutrophil priming unlikely, improving mass assays and Ca²⁺-imaging techniques may in future further

clarify the role of these mediators, and the elucidation of other routes of Ca^{2+} -mobilisation may also be informative.

Chapter 7: The Roles of Phosphatidylinositol 3,4,5-trisphosphate and Ceramide in Neutrophil Priming by $\text{TNF}\alpha$

7.1 Introduction

In recent years it has become established that a wide range of agonists can stimulate a rapid and dramatic accumulation of the novel phospholipid phosphatidylinositol 3,4,5-trisphosphate ($\text{PtdIns}(3,4,5)\text{P}_3$) in their target cells (Traynor-Kaplan et al., 1988 and 1989, Auger et al., 1989, Stephens et al., 1991, Cantley et al., 1991, Downes et al., 1991). This event is not driven by activation of other known signalling pathways (Downes et al., 1991, Traynor-Kaplan et al., 1989, Jackson et al., 1992); however, many of the agonists which cause accumulation of $\text{PtdIns}(3,4,5)\text{P}_3$ also drive the translocation of a phosphoinositide 3-hydroxy kinase (PI3K) activity, which can synthesise $\text{PtdIns}(3,4,5)\text{P}_3$ from $\text{PtdIns}(4,5)\text{P}_2$ in vitro, into receptor-associated signalling complexes (Schlessinger and Ullrich, 1992, Corey et al., 1993). These observations led to the hypothesis that $\text{PtdIns}(3,4,5)\text{P}_3$ could function as a second messenger.

PI3K was initially characterised by means of its association with, and tyrosine phosphorylation by, receptor or *src*-associated protein tyrosine kinases (PTKs), and was found to comprise an 85 kDa regulatory subunit (Escobedo et al., 1991, Otsu et al., 1991,) and a tightly associated 110 kDa catalytic subunit (Carpenter et al., 1990). However, in human neutrophils, rapid accumulation of $\text{PtdIns}(3,4,5)\text{P}_3$ has been demonstrated in response to fMLP (Traynor-Kaplan et al., 1989), PAF and ATP (Stephens et al., 1993a and 1993b), agents which transduce their signals via heterotrimeric G-proteins; $\text{PtdIns}(3,4,5)\text{P}_3$ accumulation seen following the application of these agonists was sensitive to pertussis toxin. Furthermore, Vlahos and Matter (1992)

demonstrated that, in contrast to growth factor signal transduction systems, the activation of PI3K by fMLP in neutrophils does not require its tyrosine phosphorylation. Stephens et al. (1994) have subsequently shown that, in the U937 macrophage cell line, most, if not all ATP-stimulated PtdIns(3,4,5)P₃ accumulation is driven by the activation of a distinct PI3K that is sensitive to G $\beta\gamma$ subunits but insensitive to PTK-dependent activation. More recently still, Stoyanov et al. (1995) have cloned (from a human bone marrow cDNA library) a unique enzyme with G-protein-activated PI3K activity, designated p110 γ , which does not bind p85 and has no sequence homology to previously isolated PI3K subunits. This recently characterised enzyme may be responsible for the majority of PtdIns(3,4,5)P₃ accumulation in stimulated human neutrophils.

Studies with cells expressing mutant growth factor receptors have implicated PI3K in the regulation of mitogenesis (Fantl et al., 1992); however, PI3K activity is also stimulated in a number of non-proliferating cells including neutrophils (Traynor-Kaplan et al., 1989) PC12 cells (Kimura et al., 1994) and platelets (Kucera and Rittenhouse, 1990), suggesting additional roles for this enzyme. Further clarification of the importance of this signalling pathway was achieved when the fungal metabolite wortmannin was shown to be a potent non-competitive inhibitor of PI3K at low (nM) concentrations (Arcaro and Wymann, 1993, Okada et al., 1994b and Thelen et al., 1994). Wortmannin abolished the fMLP-triggered respiratory burst in neutrophils (Baggiolini et al., 1987); indeed, two separate pathways, one Ca²⁺-sensitive and the other wortmannin-sensitive, were found to be obligatory in fMLP-stimulated O₂⁻ generation (Dewald et al., 1988; see Figure 7.1). Since PtdIns(3,4,5)P₃ accumulation appears to be vital in signalling the fMLP-stimulated respiratory burst, we decided to investigate its potential role in neutrophil priming. Since PAF (Stephens et al., 1993a)

Figure 7.1

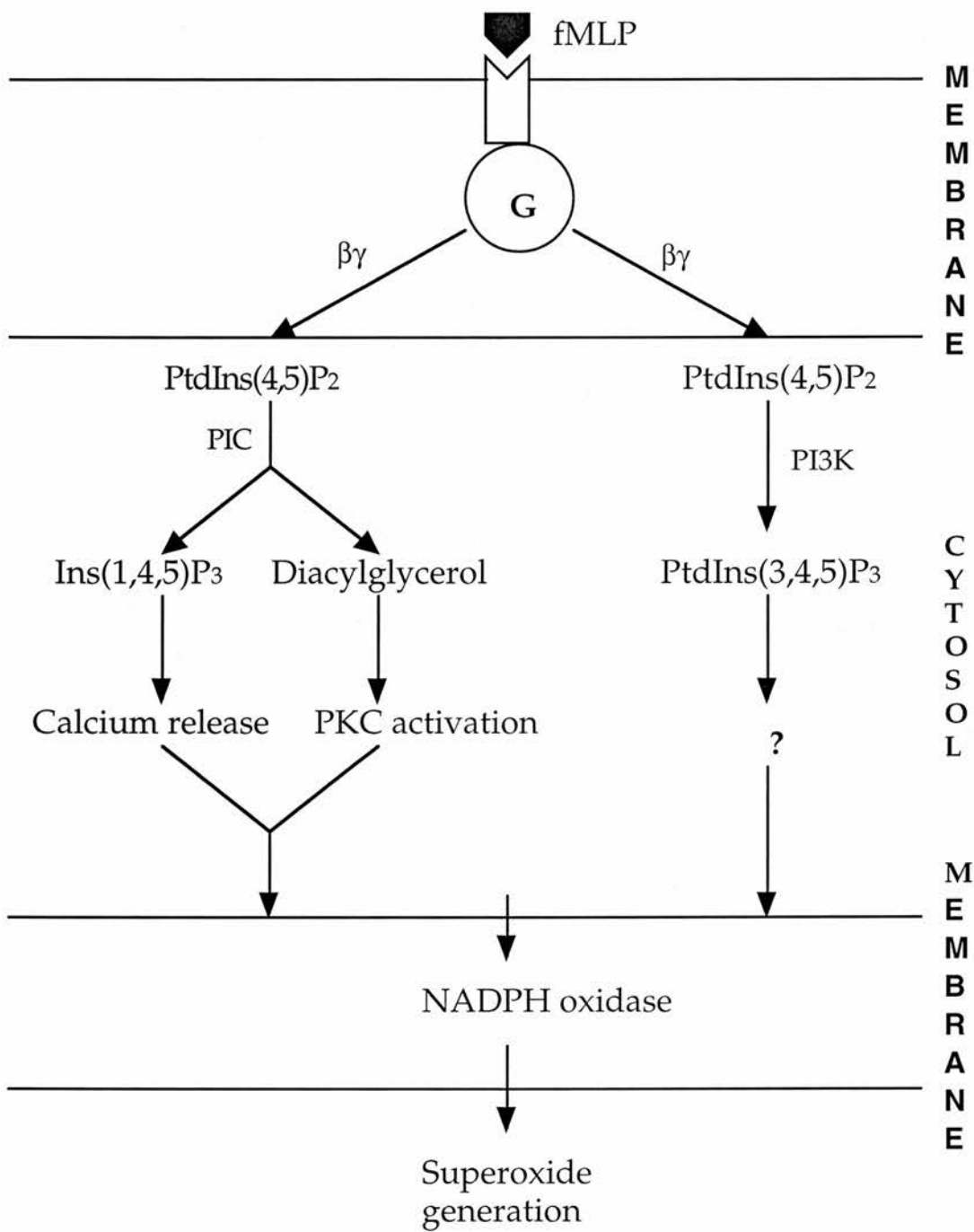


Figure 7.1 Inositol lipid signalling pathways involved in O_2^- generation

and LPS (L. Stephens, personal communication) elevate neutrophil PtdIns(3,4,5)P₃ directly, we elected to study the effects of priming with TNF α (which has no overt effect on neutrophil PI3K activity; L. Stephens, personal communication) on fMLP-stimulated human neutrophil PtdIns(3,4,5)P₃ accumulation.

During the course of these studies, ceramide (generated by the action of the enzyme sphingomyelinase on the membrane lipid sphingomyelin) was also identified as a key element in the TNF α -generated signal transduction cascade (Kim et al., 1991, Dressler et al., 1992). It has been suggested that at least in some cell types, ceramide mediates many of the effects of TNF α , including cell growth and differentiation (Olivera et al, 1992), apoptosis in HL60 cells (Obeid et al, 1993), and stimulation of a ceramide-activated protein kinase (Dressler et al., 1992). We therefore investigated the role of ceramide in neutrophil priming utilising the cell-permeable ceramide analogue C₆-ceramide, and also exogenous sphingomyelinase to generate intracellular ceramide.

7.2 Effects of C₆-ceramide and Sphingomyelinase on Human Neutrophil O₂⁻ Generation

It can be seen from Figure 7.2 that addition of the cell-permeable ceramide analogue C₆-ceramide (30 μM for 30 min at 37°C) produced no discernible direct or priming effect on O₂⁻ release; neutrophils from the same donor incubated in parallel with TNFα as a positive control exhibited a classic augmentation of the O₂⁻ response to fMLP. In a separate series of experiments C₂- and C₆-ceramide were shown to mimic the effects of TNFα in inducing apoptosis in HL-60 cells, although again these agents did not affect the rate of constitutive apoptosis in neutrophils (M. Lawson and J. Murray, personal communication). The slight apparent effect of sphingomyelinase on O₂⁻ generation (both direct and fMLP-stimulated) was in fact attributable to its vehicle (50% glycerol/PBS, 50 mM Tris-HCl, pH 7.5). It was therefore concluded that ceramide does not mediate the effect of TNFα in the priming of human neutrophils, in agreement with data obtained by Yanaga and Watson (1995).

7.3 Effects of Wortmannin on Neutrophil O₂⁻ Generation and Shape-Change

At low (nM) concentrations, the fungal metabolite wortmannin is thought to be a specific and irreversible inhibitor of PI3K (Arcaro and Wymann, 1993). Preincubation of neutrophils with 100 nM wortmannin for 30 min at 37°C completely abolished fMLP-stimulated O₂⁻ generation by both unprimed and TNFα-primed (200 U/ml, 30 min, 37°C) neutrophils (Figure 7.3.A). That wortmannin is not acting as an oxidase inhibitor is confirmed by the finding that the oxidative burst in response to PMA was unaffected or in fact slightly augmented (Figure 7.3.A). In contrast, wortmannin exerted little effect on

Figure 7.2

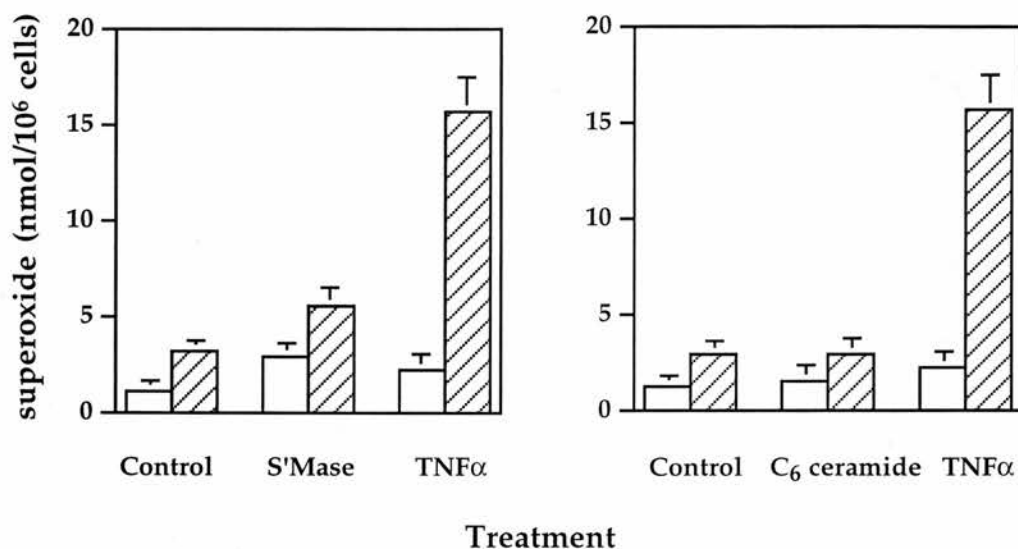


Figure 7.2 Effects of Sphingomyelinase, C₆-ceramide and TNF α on Human Neutrophil Superoxide Generation

Human neutrophils (10^6 in 90 μ l PBS with calcium and magnesium) were incubated at 37°C with neutral sphingomyelinase (S'Mase) 200 U/ml, C₆-ceramide 30 μ M, TNF α 200 U/ml or the appropriate vehicles for 30 min prior to the addition of pre-warmed cytochrome C (final concentration 1.2 mg/ml) and fMLP (final concentration 100 nM, hatched bars) or vehicle (open bars) in a final volume of 1 ml. SOD 375 U was included in one of each set of quadruplicate incubations. After 15 min cells were pelleted at 4°C and the optical density of the supernatants determined at 550 nm by scanning spectrophotometry; O₂⁻ generation was calculated as described in (2.3). Data represent mean \pm sem of 3 separate experiments, each performed in triplicate.

Figure 7.3

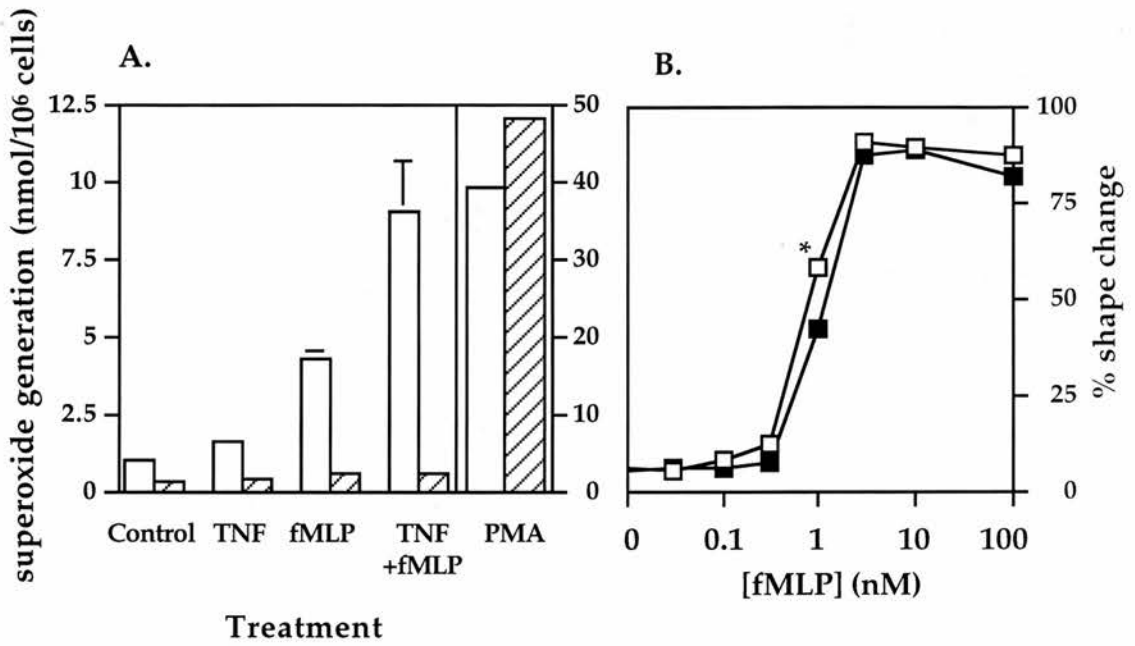


Figure 7.3 Effects of Wortmannin on O₂⁻ Generation and Shape Change

A. Neutrophils (10⁷/ml) were incubated in the presence (hatched bars) or absence (open bars) of 100 nM wortmannin for 30 min prior to addition of TNF α (200 U/ml, 30 min) or PBS and stimulation (10 min) with 100 nM fMLP, 10 ng/ml PMA or appropriate buffer (controls). Superoxide generation was measured by the superoxide dismutase-inhibitable reduction of cytochrome C as previously described (2.3), n=3.

B. Neutrophils (5x10⁶/ml) were incubated in the presence (closed symbols) or absence (open symbols) of 100 nM wortmannin for 30 min prior to treatment with fMLP 0-100 nM; reactions were terminated at 10 min by fixation with an equal volume of 0.5% glutaraldehyde. Shape-change was assessed under light microscopy as described (2.4). Data are mean \pm sem of n=3 duplicate determinations; all error bars were <10% of the mean and lie within the data points. * p<0.05 for presence versus absence of wortmannin.

fMLP-induced shape-change, inhibiting this response only when 1 nM fMLP was used as the stimulus (Figure 7.3.B). Wortmannin (100 nM) did not affect cell viability as assessed by trypan blue exclusion (data not shown).

7.4 Priming of Neutrophils in Phosphate-free Buffer

To optimise labelling of neutrophil phospholipids with [^{32}P] P_i , a prolonged (70 min) incubation of cells with the isotope in phosphate-free medium was utilised. To confirm that priming could occur under these conditions, neutrophils underwent a 70 min incubation in sterile, phosphate-free medium (110 mM NaCl, 10 mM KCl, 1 mM MgCl_2 , 10 mM glucose, 30 mM HEPES, 1.5 mM CaCl_2 , pH 7.4, custom supplied by GIBCO) prior to priming with $\text{TNF}\alpha$ (200 U/ml, 30 min) and measurement of fMLP-stimulated O_2^- release. As illustrated (Figure 7.4), a competent priming response was still observed in the phosphate-free medium even with the inclusion of an additional 70 min incubation step, and cells not treated with $\text{TNF}\alpha$ produced little O_2^- in response to 100 nM fMLP, thereby validating the labelling procedure.

7.5 fMLP-stimulated $\text{PtdIns}(3,4,5)\text{P}_3$ Accumulation in Unprimed and $\text{TNF}\alpha$ -primed Human Neutrophils

$\text{PtdIns}(3,4,5)\text{P}_3$ determinations were carried out in unprimed and $\text{TNF}\alpha$ -primed neutrophils at 10 s and 1 min following stimulation with 100 nM fMLP. Logistics (the cell numbers required, and the level of radioactivity involved) dictated that a single time-point only was examined in each experiment. At 10 s after the addition of fMLP (Figures 7.5 and 7.6.A) a 1.4-fold enhancement of stimulated $\text{PtdIns}(3,4,5)\text{P}_3$ accumulation in primed versus unprimed cells was demonstrated. However at 1 min a 6.2-fold increase in $\text{PtdIns}(3,4,5)\text{P}_3$ was seen in primed cells stimulated with fMLP

Figure 7.4

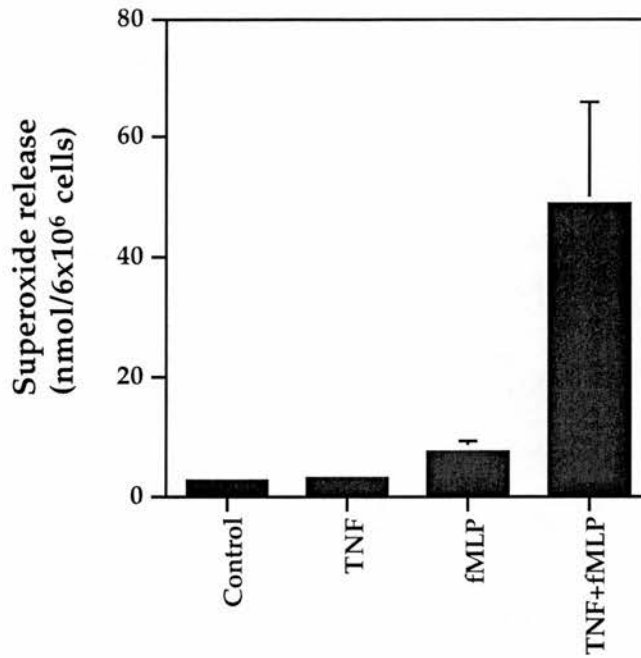


Figure 7.4 Priming of Human Neutrophils in Phosphate-free Medium

10^8 neutrophils suspended in 2.5 ml phosphate-free buffer were incubated in a shaking water bath for 70 min at 37°C, washed twice and resuspended in 1.2 ml phosphate-free medium. 100 μ l aliquots of cells were further incubated with TNF α (200 U/ml, added in 40 μ l) or buffer control for 30 min and stimulated with 100 nM fMLP (40 μ l). O₂⁻ release was quantified by the superoxide dismutase-inhibitable reduction of cytochrome C as previously described (2.3).

Figure 7.5

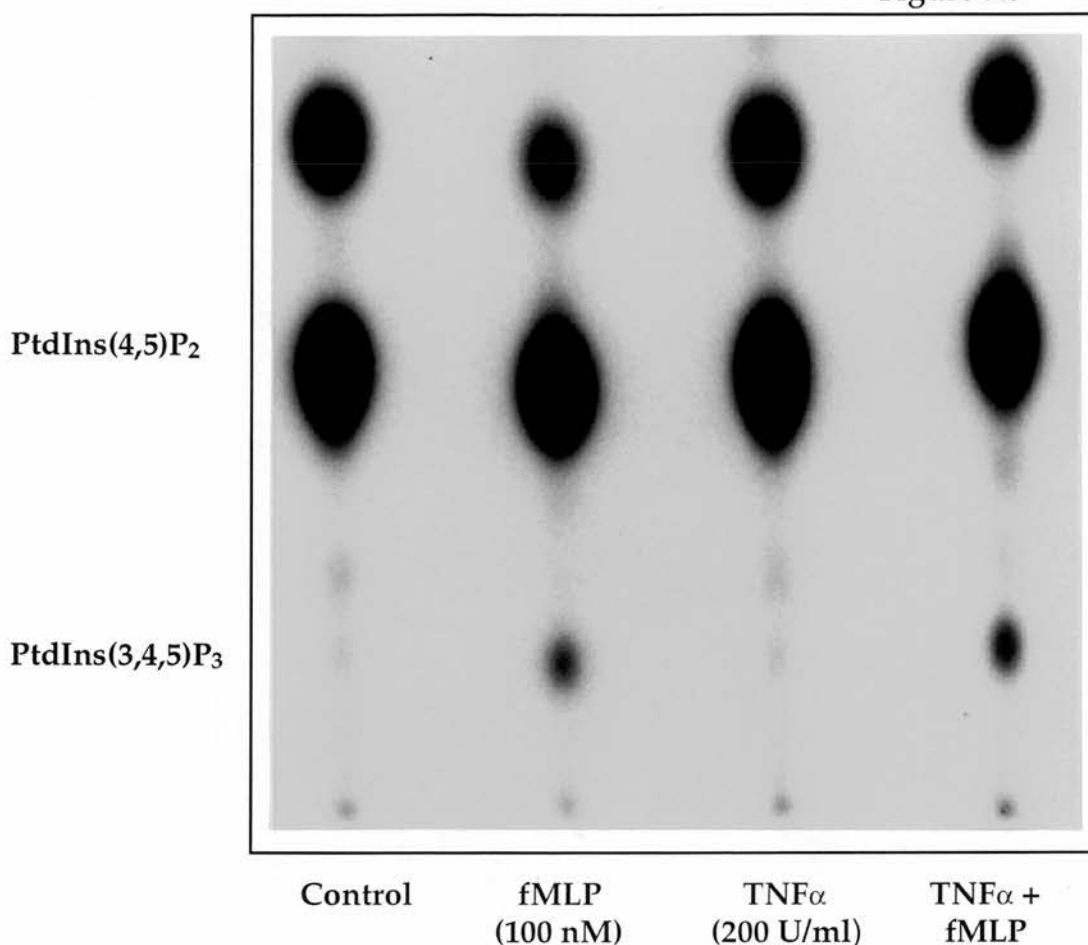


Figure 7.5 Effects of TNF α on fMLP-stimulated (10 s) PtdIns(3,4,5)P₃ Accumulation in Human Neutrophils: Autoradiograph of TLC Plate

Human neutrophils (10^8 in 2.5 ml phosphate-free buffer) were labelled with 2 mCi/ml [32 P]P_i (70 min, 37°C), washed twice and resuspended in 1.2 ml phosphate-free buffer. 100 μ l aliquots of labelled cells were incubated in the presence or absence of 200 U/ml TNF α (added in 40 μ l) for 30 min prior to stimulation with fMLP 100 nM (40 μ l). Reactions were quenched at 10 s by the addition of 675 μ l methanol/chloroform (2:1, v/v) and lipids were extracted and deacylated as described (2.8.2). The deacylated lipids extracts were dried and redissolved in 2 μ l 20 mM HCl, 1 mM K₂PO₄ and chromatographed on PEI-cellulose plates; the plates were developed in 0.48 M HCl and the radioactivity quantified by means of a phosphoimager.

Figure 7.6

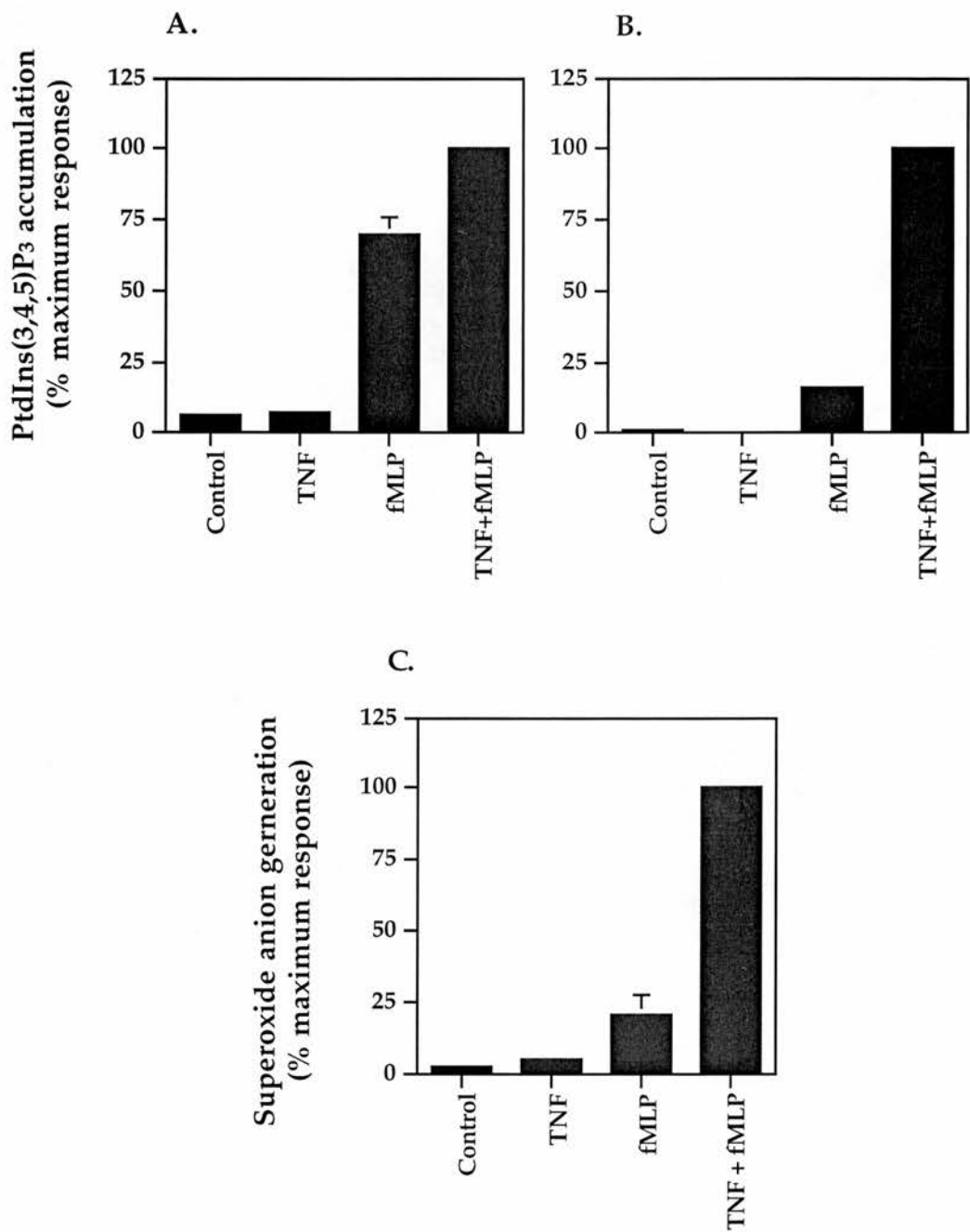


Figure 7.6 fMLP-stimulated PtdIns(3,4,5)P₃ Accumulation In Unprimed and TNF α -primed Human Neutrophils

Figure legend overleaf.

Figure Legend for Figure 7.6

Figure 7.6 fMLP-stimulated PtdIns(3,4,5)P₃ Accumulation In Unprimed and TNF α -primed Human Neutrophils

A and B. Human neutrophils (10^8 in 2.5 ml phosphate-free buffer) were labelled with 2 mCi/ml [³²P]P_i (70 min, 37°C), washed twice and resuspended in 1.2 ml phosphate-free buffer. 100 μ l aliquots of labelled cells were incubated in the presence or absence of 200 U/ml TNF α (added in 40 μ l) for 30 min prior to stimulation with fMLP 100 nM (40 μ l). Reactions were quenched at 10 s (A) or 60 s (B) by the addition of 675 μ l methanol/chloroform (2:1, v/v) and lipids were extracted and deacylated as described (2.8.2). The deacylated lipids were dried and analysed by TLC or HPLC as detailed in (2.8.2). Data (mean \pm sem of triplicate determinations for n=3 (A) or n=2 (B)) are expressed as % maximum response.

C. For the n=5 experiments represented by (A) and (B), neutrophils underwent the labelling, priming and stimulation procedures and were subsequently analysed for superoxide release by the superoxide dismutase-inhibitable reduction of cytochrome C (2.3). Data (mean \pm sem, n=5 experiments each performed in triplicate) are expressed as % maximum response.

above that seen in unprimed stimulated cells (Figure 7.6.B); the level of accumulation in primed and stimulated cells was >100-fold that seen in control cells. $\text{TNF}\alpha$ alone did not affect $\text{PtdIns}(3,4,5)\text{P}_3$ detection. Superoxide anion release was measured simultaneously to confirm adequacy of priming (Figure 7.6.C).

7.6 Effects of $\text{TNF}\alpha$ and fMLP on $[^{32}\text{P}]$ Phosphoinositides.

Incubation of $[^{32}\text{P}]\text{P}_i$ -labelled neutrophils with 200 U/ml $\text{TNF}\alpha$ for 30 min resulted in an increase in $[^{32}\text{P}]\text{PtdIns}4\text{P}$ (Tables 7.1 and 7.2); no consistent effect on $[^{32}\text{P}]\text{PtdIns}(4,5)\text{P}_2$, the immediate precursor of $\text{PtdIns}(3,4,5)\text{P}_3$, was observed. Most strikingly, the changes in $[^{32}\text{P}]\text{PtdIns}(3,4,5)\text{P}_3$ were closely paralleled by changes in its immediate metabolite, $[^{32}\text{P}]\text{PtdIns}(3,4)\text{P}_2$. Both phospholipids were virtually undetectable in control and $\text{TNF}\alpha$ -treated cells; following fMLP stimulation, at 10 sec near maximal elevation was observed in unprimed cells whilst at 60 s the accumulation of both phosphoinositides was much greater in primed cells (6.2-fold and 7.6-fold respectively); accumulation in primed cells was far greater at 1 min than at 10 sec, whilst levels in unprimed cells were similar at the 2 time-points. Accumulation of $\text{PtdIns}3\text{P}$ was also increased in primed versus unprimed cells 1 min after stimulation, with only a slight effect at 10 s. At 1 min, depletion of $[^{32}\text{P}]\text{PtdIns}(4,5)\text{P}_2$ was much greater in primed, stimulated cells, consistent with sustained PI3K activity to form $[^{32}\text{P}]\text{PtdIns}(3,4,5)\text{P}_3$.

7.7 Effects of $\text{TNF}\alpha$ and fMLP on Neutrophil PI3K Activity in Anti-phosphotyrosine Immunoprecipitates

To further characterise the enhanced accumulation of $\text{PtdIns}(3,4,5)\text{P}_3$ seen in $\text{TNF}\alpha$ -primed human neutrophils 1 min following stimulation with fMLP,

Table 7.1

	PtdIns4P	PtdIns(4,5)P ₂	PtdIns(3,4)P ₂	PtdIns3P	PtdIns(3,4,5)P ₃
Control	15897±1055	41027±2232	29±6	541±43	96±16
TNF	23626±520	49091±926	50±3	775±7	118±38
fMLP	15119±627	32668±1775	397±27	574±51	1682±97
TNF+fMLP	22188±1013	41210±2080	554±43	824±77	2661±107

Table 7.1 Effects of TNF α on fMLP-stimulated (10 s) Changes in Human Neutrophil Phosphoinositides.

[³²P]P_i-labelled human neutrophils were washed twice and resuspended at 6x10⁷/ml in phosphate-free buffer. 100 μ l aliquots of labelled cells were incubated in the presence or absence of 200 U/ml TNF α (added in 40 μ l) for 30 min prior to stimulation with fMLP 100 nM (40 μ l). Reactions were quenched at 10 s by the addition of 675 μ l methanol/chloroform (2:1, v/v) and lipids were extracted and deacylated as described (2.8.2). The [³²P]glycerophosphoesters were resolved by anion-exchange chromatography on a Partisphere SAX column by Dr. L. Stephens (Department of Signalling, Babraham Institute, Cambridge) as described (2.8.2). Data (d.p.m.) are mean \pm sem from a representative experiment, all points in triplicate

Table 7.2

	PtdIns4P	PtdIns(4,5)P ₂	PtdIns(3,4)P ₂	PtdIns3P	PtdIns(3,4,5)P ₃
Control	9567±813	24062±830	24±5	252±49	31±3
TNF	13298±689	22814±2081	38±4	381±28	28±3
fMLP	10803±242	21972±189	308±30	308±10	535±35
TNF+fMLP	11890±451	15702±353	2349±204	629±44	3316±354

Table 7.2 Effects of TNF α on fMLP-stimulated (60 s) Changes in Human Neutrophil Phosphoinositides

[³²P]P_i-labelled human neutrophils were washed twice and resuspended at 6x10⁷/ml in phosphate-free buffer. 100 μ l aliquots of labelled cells were incubated in the presence or absence of 200 U/ml TNF α (added in 40 μ l) for 30 min prior to stimulation with fMLP 100 nM (40 μ l). Reactions were quenched at 60 s by the addition of 675 μ l methanol/chloroform (2:1, v/v) and lipids were extracted and deacylated as described (2.8.2). The [³²P]glycerophosphoesters were resolved by anion-exchange chromatography on a Partisphere SAX column by Dr. L. Stephens (Department of Signalling, Babraham Institute, Cambridge) as described (2.8.2). Data are d.p.m., mean \pm sem, n=2 triplicate values.

PI3K activity in PY20 antiphosphotyrosine immunoprecipitates was determined by Dr L. Stephens, Department of Signalling, Babraham Institute, Cambridge. Figure 7.9 illustrates that a 1.7-fold increase in primed versus unprimed cells was detected; simultaneous measurement of fMLP-stimulated O_2^- release revealed an amplification of greater than 13-fold in primed versus unprimed cells. Interestingly, a slight decrease in PI3K activity below control values was noted in response to $TNF\alpha$ alone, with fMLP alone having not significant effect.

Figure 7.7

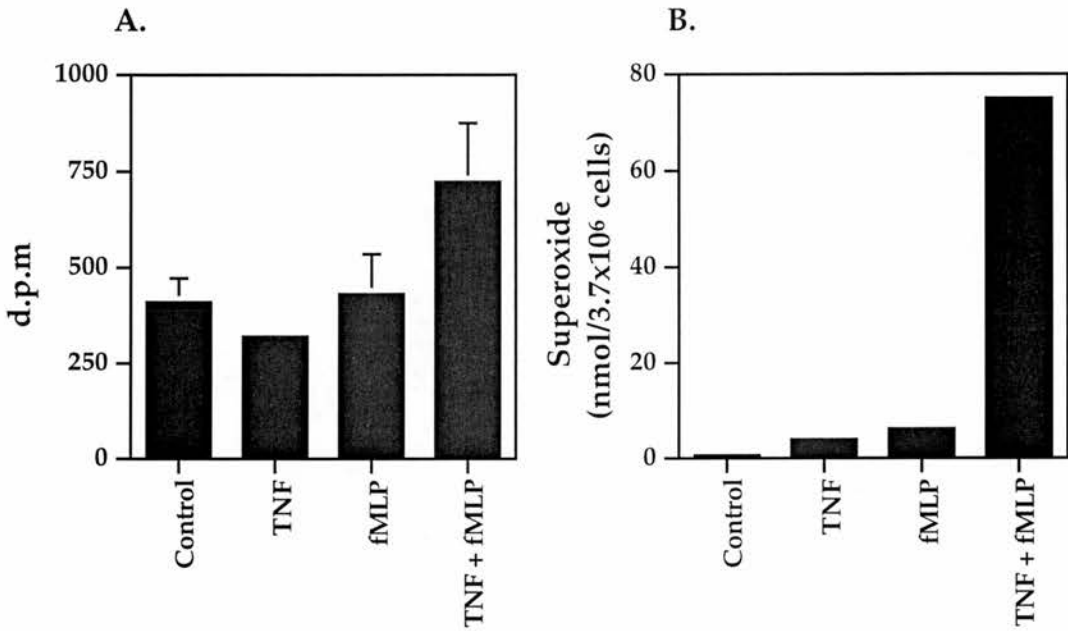


Figure 7.7 Effects of TNF α and fMLP on Neutrophil PI3K Activity in Anti-phosphotyrosine Immunoprecipitates

A). Human neutrophils (3×10^7 /ml) were incubated in the presence or absence of TNF α (200 U/ml) for 30 min and stimulated with fMLP 100 nM or vehicle for 60 s prior to pelleting and snap-freezing in liquid nitrogen. The PI3K activity in antiphosphotyrosine immunoprecipitates was quantified by Dr L. Stephens according to the method described in (2.8.3). Data represent the amount of [32 P] incorporated into PtdIns3P during the assay and are the mean of $n=2$ determinations performed in triplicate.

B). Simultaneously, neutrophils from the same donors were primed with TNF α 200 U/ml and stimulated with fMLP 100 nM; the release of O_2^- was quantified by the superoxide dismutase-inhibitable reduction of cytochrome

C. Data are the mean of $n=3$ determinations performed in triplicate.

7.8 Discussion

The key finding presented in this chapter is that priming with TNF α augmented the accumulation of PtdIns(3,4,5)P₃ generated in human neutrophils in response to fMLP stimulation. This effect was relatively small at an early time-point (10 s) following the addition of fMLP (fMLP alone 70% \pm 5.6% of the PtdIns(3,4,5)P₃ accumulation seen in primed and stimulated cells) but by 1 min after fMLP stimulation PtdIns(3,4,5)P₃ accumulation was enhanced by 620% in primed cells, and the value for primed and stimulated cells was approximately 100 times that seen in control unprimed, unstimulated cells. The effect of fMLP alone in these experiments is comparable to that reported by other investigators (e.g. Traynor-Kaplan et al., 1989); no previous studies of the possible role of 3-phosphorylated inositol phospholipids in neutrophil priming have been reported. The finding that the magnitude and duration of the PtdIns(3,4,5)P₃ were greatly increased in TNF α -primed cells suggests a possible role in signalling for the primed respiratory burst.

The fact that fMLP-stimulated PtdIns(3,4,5)P₃ accumulation in neutrophils is pertussis toxin-sensitive but resistant to tyrosine kinase inhibitors (Stephens et al., 1993b, Corey et al., 1993) and associated with little (Stephens et al., 1993b) or no (Vlahos and Matter, 1992) increase in PI3K activity detected in anti-phosphotyrosine antibody immunoprecipitates, indicates that the classical p85/p110 enzyme activated by receptor or src-type tyrosine kinases may not be the principal PI3K isoform associated with the fMLP-driven PtdIns(3,4,5)P₃ response in these cells. We have confirmed that neither fMLP TNF α alone induced a significant increase in the PI3K activity associated with antiphosphotyrosine immunoprecipitates; in fact, treatment with TNF α

alone resulted in a slight fall in the baseline PI3K activity present in anti-phosphotyrosine immunoprecipitates; a similar effect has been reported with fMLP alone (in THP-1 cells: Okada et al., 1996) and with thrombin (in 1321N1 cells: Batty and Downes, 1996) but its significance is unclear. The increase in the anti-phosphotyrosine immunoprecipitable PI3K activity seen in TNF α -primed and stimulated cells versus unprimed stimulated cells (1.7-fold) is slight in comparison to the corresponding increases in PtdIns(3,4,5)P₃ detection (6.2-fold) and O₂⁻ generation (13.3-fold); comparison of control (unprimed, unstimulated) cells with primed and stimulated cells reveals an even greater gulf between increase in the PI3K activity present in immunoprecipitates (1.7-fold increase) and elevation of PtdIns(3,4,5)P₃ (approximately 100-fold increase) or augmentation of O₂⁻ generation (>1000-fold increase). Thus, although a small contribution from the recruitment and activation of a tyrosine kinase-regulatable p85/p110 PI3K cannot be excluded, it seems likely that the majority of the PtdIns(3,4,5)P₃ generated in stimulated neutrophils is the product of a different PI3K isoform.

Stephens et al. (1994) first described the activation by G-protein $\beta\gamma$ -subunits of a PI3K isoenzyme immunologically and chromatographically distinct from the well-characterised p85/p110 enzyme; the identification and cloning of a novel catalytic subunit of PI3K (p110 γ) that is activated by both α and $\beta\gamma$ -subunits of heterotrimeric G-proteins was subsequently reported (Stoyanov et al., 1995). More recently, Okada et al. (1996) showed that insulin and fMLP can exert a synergistic effect on PtdIns(3,4,5)P₃ formation in THP1 cells, suggesting that there is cross-talk between the tyrosine phosphorylation-dependent transduction pathway and the G-protein-dependent pathway; this was confirmed in a cell-free system. The authors also separated two PI3K activities, one responsive to $\beta\gamma$ -subunits alone and the other activated synergistically by $\beta\gamma$ -subunits and by a tyrosine-

phosphorylated peptide based on the insulin receptor-substrate-1. These data may well be pertinent to neutrophils, since elevations of neutrophil $\text{PtdIns}(3,4,5)\text{P}_3$ have been documented in response to GM-CSF in a genistein-sensitive but pertussis toxin-insensitive fashion (Corey et al., 1993). However, although $\text{TNF}\alpha$ can stimulate tyrosine phosphorylation of neutrophil proteins (Berkow and Dodson, 1988, Lloyds et al., 1995), our data demonstrate that $\text{TNF}\alpha$ did not significantly affect $\text{PtdIns}(3,4,5)\text{P}_3$ accumulation at 30 min or directly stimulate PI3K activity in PY20 immunoprecipitates; Corey et al. (1993) also reported that incubation of neutrophils with $\text{TNF}\alpha$ 1000 U/ml for 3 min did not lead to significant $\text{PtdIns}(3,4,5)\text{P}_3$ elevation. Additionally, in the experiments described by Okada et al. (1996), the two agonists (fMLP and insulin) were added simultaneously. This suggests that the situation in $\text{TNF}\alpha$ -primed neutrophils is not directly analogous to their system, in that $\text{TNF}\alpha$ is not synergistically activating one or more isoforms of PI3K with fMLP. Since the enhanced accumulation of $\text{PtdIns}(3,4,5)\text{P}_3$ did not appear to reflect increased $\text{PtdIns}(4,5)\text{P}_2$ substrate availability or decreased $\text{PtdIns}(3,4,5)\text{P}_3$ -5 phosphatase activity (Tables 7.1 and 7.2), it is likely that $\text{TNF}\alpha$ acts directly on PI3K itself, or at a proximal step in the signal transduction pathway.

The fungal metabolite wortmannin has been shown to inhibit PI3K (Arcaro and Wymann, 1993, Okada et al., 1994b, Stoyanov et al., 1995) by binding irreversibly to the enzyme (Thelen et al., 1994). This interaction has been shown to inhibit respiratory burst activity and granule release induced by fMLP, C5a, PAF and LTB_4 (but not PMA) without influencing agonist-induced Ca^{2+} transients or directly affecting protein kinase C, the NADPH-oxidase or the process of granule exocytosis (Baggiolini et al., 1987, Dewald et al., 1988). Unfortunately, as wortmannin abolished O_2^- generation in response to fMLP in unprimed as well as primed cells (Figure 7.3), its

usefulness in investigating any selective role for PI3K in neutrophil priming is severely limited. Additionally, concerns have been raised regarding the specificity of this inhibition, since wortmannin has been reported to inhibit other enzymes such as myosin light chain kinase (Nakashini et al., 1992), phospholipase C (Nakashini et al., 1994, Bonser et al., 1991), phospholipase D (Bonser et al., 1991) and phospholipase A₂ (Cross et al., 1995); however in all but the last report the IC₅₀ values for inhibition were considerably higher than the IC₅₀ reported for inhibition of PI3K by wortmannin (low nanomolar). A second PI3K inhibitor, LY294002, has more recently been characterised (Vlahos et al., 1995); its effects on neutrophils are similar to those of wortmannin, but as yet data concerning its specificity are lacking. Inhibition of PI3K activity has been shown to compromise many different cellular activities, including cell growth and mitogenesis (Auger et al., 1989, Cantley et al., 1991), membrane ruffling (Wennström et al., 1994), insulin-induced glucose transport (Okada et al., 1994a), early endosome fusion (Jones and Clague, 1995) and the neutrophil respiratory burst (Dewald et al., 1988). The downstream targets of PtdIns(3,4,5)P₃ responsible for exerting these varying functions are only just beginning to be elucidated. Studies in a variety of cell lines, utilising receptor mutations and PI3K inhibitors, have suggested that PI3K may lie upstream of several protein kinase cascades, including p70 S6 kinase (Chung et al., 1994, Baxter et al., 1995), the MAPK pathway activated by PDGF, insulin and IL-2 (Baxter et al., 1995, Welsh et al., 1994, Cross et al., 1995, Karnitz et al., 1995) and the serine-threonine kinase Akt-1 (Franke et al., 1995, Kohn et al., 1995); however, the relevance of these observations to O₂⁻ generation in neutrophils is at present unclear. Activation of Ca²⁺-independent PKC isoforms by PtdIns(3,4,5)P₃ has also been demonstrated (Nakanishi et al., 1992, Toker et al., 1994); as phosphorylation of P47^{phox} by PKC is involved in activation of the

respiratory burst, this is a potential mechanism by which PI3K could influence the neutrophil respiratory burst. However, activation of PKC by alternative routes (Ca^{2+} and DAG) also occurs during fMLP stimulation, hence PKC is unlikely to be the only effector of $\text{PtdIns}(3,4,5)\text{P}_3$ involved. A further downstream target of $\text{PtdIns}(3,4,5)\text{P}_3$ which is more likely to be physiologically relevant is the small G-protein p21^{rac} , whose translocation to the neutrophil membrane is essential for activation of the respiratory burst (Quinn et al., 1993, Abo et al., 1994). Hawkins et al. (1995) have demonstrated that p21^{rac} activation lies downstream of PI3K activation in the mediation of PDGF-induced membrane ruffling. In resting human neutrophils, all the p21^{rac} present in the cytosol exists in an inactive state complexed to GDI, but this interaction was shown to be disrupted by various lipid mediators including arachidonic acid and phosphatidylinositols (Chuang et al., 1993). Furthermore, in bcr-null mutant mice (bcr limits p21^{rac} activation by enhancing the intrinsic rate of GTP hydrolysis), a threefold increase in p21^{rac} translocation to the neutrophil membrane was observed, and neutrophils from these mice generated more O_2^- in response to agonists than did those from wild-type mice (Voncken et al., 1995). Thus it is possible that the exaggerated $\text{PtdIns}(3,4,5)\text{P}_3$ response seen on stimulation of $\text{TNF}\alpha$ -primed human neutrophils is translated into enhanced p21^{rac} dissociation from GDI and subsequent membrane translocation, with concomitant activation of the NADPH oxidase.

The cell-permeable ceramide analogue C_6 -ceramide and exogenous sphingomyelinase failed to prime or stimulate the neutrophil NADPH oxidase (Figure 7.2), suggesting that elevation of ceramide alone is not sufficient to elicit either of these response. However, these data do not exclude a permissive role for ceramide in priming; in a recent paper, Higuchi et al. (1996) demonstrated that ceramide analogues and exogenous

sphingomyelinase did not influence apoptosis in human myeloid leukaemia M1-1a cells, but that inhibition of acid sphingomyelinase blocked the pro-apoptotic effect of $\text{TNF}\alpha$; furthermore, this block could be overcome by the inclusion of ceramide analogues in the incubation. The role of ceramide as a second messenger for other agonists such as LPS and PAF is completely unknown, and detailed studies await the general availability of a specific sphingomyelinase inhibitor.

In summary, priming of human neutrophils with $\text{TNF}\alpha$ leads to an augmented and prolonged fMLP-stimulated $\text{PtdIns}(3,4,5)\text{P}_3$ response; the accompanying increase of anti-phosphotyrosine immunoprecipitable PI3K activity is of much smaller magnitude, suggesting that activation of the $\beta\gamma$ -regulated PI3K isozyme may be largely responsible for the enhanced response.

CHAPTER 8: SUMMARY

This thesis has focused on the signal transduction mechanisms involved in neutrophil priming, the process by which resting neutrophils achieve a state of preactivation associated with greatly augmented responsiveness on subsequent stimulation.

The *in vivo* relevance of priming has been established by the detection of priming agents in the circulation of many patients with sepsis (Parsons et al., 1989, Sun et al., 1990), by the detection of primed neutrophil subpopulations in patients with inflammatory disease states (Chollet-Martin et al., 1992, Bass et al., 1986) and by the enhanced release of ROI from neutrophils observed following infusion of priming agents into healthy volunteers (Wewers et al., 1990, Van der Poll et al., 1992) or laboratory animals (e.g. Mayer and Spitzer, 1991). However, some procedures used to isolate pure populations of neutrophils from whole blood have been shown to effect a degree of priming (Haslett et al., 1985), perhaps by the exposure of cells to trace concentrations of LPS contaminating laboratory reagents. The *in vitro* study of priming mechanisms necessitates the isolation of purified but unprimed neutrophils; we have found that this ideal situation is most closely approached using neutrophils separated on plasma/Percoll gradients (Haslett et al., 1985); when this preparative method was used, neutrophils produced only minimal quantities of O_2^- unless they were first incubated with a priming agent (e.g. Figures 3.3-3.5).

The wide range of priming agents so far identified encompasses bacterial products, inflammatory cytokines, pharmacological agents and the cross-linking of adhesion molecules (see Table 1.1). In this thesis, physiologically relevant inflammatory mediators - LPS, $TNF\alpha$ and PAF - have been selected

to further study priming mechanisms. LPS, the first neutrophil priming agent identified (Guthrie et al., 1984) is released from Gram-negative bacteria; in vitro, low concentrations of LPS have been shown to accentuate chemotactic factor-induced endothelial injury (Smedley et al., 1986), and in vivo low doses of LPS infused into rabbits greatly accentuate the tissue damage caused by subsequent injection of formylated peptide or complement fragments (Worthen et al., 1987). $\text{TNF}\alpha$ is produced in response to LPS, principally by cells of the monocyte-macrophage lineage, and serum levels of this cytokine correlate with a poor outcome in septic shock (Pinsky et al., 1993). Endothelial monolayers treated with thrombin prime neutrophils layered on top of them by the production of the biologically active lipid PAF (Vercellotti et al., 1989); in an in vivo study of endotoxic shock, elevated plasma levels of both $\text{TNF}\alpha$ and PAF were required to produce severe hypotension, and many of the detrimental effects of endotoxin infusion were prevented by PAF antagonists (Sun et al., 1990), further emphasising the in vivo significance of PAF as a biological mediator. PAF is expressed by stimulated endothelial cells (Vercellotti et al., 1988, Lorant et al., 1991) and may therefore modulate the function of adherent neutrophils in vivo (Lorant et al., 1993). With reference to their physiological importance and efficacy as neutrophil priming agents (Figures 3.2-3.5) these three agonists utilise widely differing transduction machinery (PAF acts via a G-protein-linked receptor and primes very rapidly, TNF interacts with a receptor trimer and induces priming in 10-30 min, and LPS complexes with LBP to activate transduction processes via CD14 but requires 60-120 min to establish the primed state).

Agonist concentrations and incubation conditions were selected to produce equivalent priming of fMLP-stimulated O_2^- release (LPS 100 ng/ml for 60-120 min, $\text{TNF}\alpha$ 200 U/ml for 30 min and PAF 100 ng/ml for 10 min).

Unfortunately, different batches of LPS (from the same supplier) were found to vary in their priming efficacy (see, for example, Figure 3.3 versus Figure 3.6); additionally, although washing procedures were standardised, it is possible that exposure of neutrophils to serum proteins during centrifugation through plasma/Percoll gradients may have affected their responses (Figure 3.2). For this reason, in some experiments 1% heat-inactivated serum was included with LPS in the incubation medium. The activity of TNF α in U/ml, rather than the concentration, was kept constant since wide variations in the activity per mg were quoted for different batches of the reagent (again from a constant supplier). When experimental protocols necessitated further manipulation of neutrophils or extra incubation steps, simultaneous determinations of fMLP-stimulated O₂⁻ release were performed to ensure that the cells were not primed by the new experimental conditions (see, for example, Figure 7.4).

Cell-cell and cell-matrix interactions provide communication between cells and their environment, and are essential to the development and maintenance of inflammatory responses. Recruitment of leukocytes to sites of inflammation and infection is dependent on the carbohydrate-binding selectin glycoproteins, the integrins and the immunoglobulin superfamily members. Priming agents have been shown to regulate neutrophil adhesion molecule expression in an agonist-specific fashion. LPS, TNF α and PAF all upregulated integrin expression and decreased neutrophil CD62-L expression (Figures 4.2-4.4); however, the effects of PAF on CD11b were seen at lower concentrations than those required to influence CD62-L (EC₅₀ 38 \pm 12 nM versus 102 \pm 23 nM respectively) whilst for LPS, the opposite was true (EC₅₀ for CD11b upregulation >204 \pm 49 ng/ml, and for CD62-L loss 53 \pm 6 ng/ml). TNF α affected CD11b and CD62L expression with fairly similar potency (EC₅₀ 138 \pm 35 U/ml versus 71 \pm 24 U/ml). For each agonist, the time-

course for modulation of adhesion molecule expression correlated closely with the time-course for augmentation of the stimulated respiratory burst (Figures 4.6 and 4.7 versus Figure 3.6). Priming agents also caused a profound upregulation of integrin function, as judged by the binding of ACLB; this event is probably as critical to neutrophil adhesion as the upregulation of integrin expression (Vedder and Harlan., 1988). Modulation of neutrophil adhesion molecule expression may represent a mechanism whereby priming agents alter subsequent responsiveness to activating stimuli, since engagement of integrins also initiates multiple signalling cascades (Clarke and Brugge, 1995), and upregulation of integrin expression may promote such signals. While this may be of limited relevance to cells maintained in suspension (as examined throughout this thesis), integrin engagement is essential for the conversion of TNF α and GM-CSF from priming agents to powerful secretagogues (Nathan et al., 1989a).

In HL-60 cells differentiated towards neutrophil maturation, the fMLP receptor is coupled to PIC via G $_{i\alpha 2}$ and G $_{i\alpha 3}$, (Uhing et al., 1987, Gierschik et al., 1989). In neutrophils, the major isoenzyme of PI3K responsible for phosphorylating PtdIns(4,5)P $_2$ to PtdIns(3,4,5)P $_3$ is coupled to G-proteins and not regulated by tyrosine kinases as is the conventional p85/p110 isoenzyme (Stephens et al., 1994, Stoyanov et al., 1995 and Figure 7.7). Furthermore, upregulation of membrane-associated G $_{i\alpha 2}$ has been demonstrated to occur on incubation of neutrophils with LPS (Yasui et al., 1992), GM-CSF (Durstin et al., 1993) and TNF α (Klein et al., 1995). In view of these findings, a detailed study to examine the relationship between G $_{i\alpha 2}$ translocation and priming by LPS, TNF α and PAF was undertaken. All three priming agents increased the detection of G $_{i\alpha 2}$ in neutrophil membranes, the time-course of this upregulation correlating closely with the time-course for augmentation of the fMLP-stimulated respiratory burst

(Figures 5.1-5.3 compared to Figure 3.6). However, the degree of upregulation of $G_{i\alpha 2}$ expression and function was far less than the corresponding upregulation of stimulated O_2^- production. Since little or no augmentation of the $\text{Ins}(1,4,5)\text{P}_3$ response to submaximal concentrations of fMLP was seen in primed cells (Chapter 6), it is unlikely that the modest degree of G-protein upregulation is relevant in this signalling pathway, although it could make a minor contribution to the augmented $\text{PtdIns}(3,4,5)\text{P}_3$ effect (see Chapter 7 and below).

Few previous studies have addressed the role of $\text{Ins}(1,4,5)\text{P}_3$ (the signalling molecule responsible for release of Ca^{2+} from intracellular stores) in neutrophil priming, although modulation of basal and peak Ca^{2+} levels has been implicated as a mechanism underlying the effects of priming agents and the Ca^{2+} transient itself is essential for secretagogue-induced activation of the respiratory burst (Forehand et al., 1989, Finkel et al., 1987, Dewald et al., 1988). A detailed investigation of the effects of priming agents on basal and fMLP-stimulated $\text{Ins}(1,4,5)\text{P}_3$ accumulation was therefore undertaken, using a mass assay to minimise the need for additional manipulation of the cells under study. fMLP (100 nM) alone induced maximal $\text{Ins}(1,4,5)\text{P}_3$ accumulation that was not augmented by preincubation under optimal priming conditions with any of the priming agents studied (Figures 6.2-6.4, 6.5 and 6.7); under identical conditions, the respiratory burst was minimal in response to fMLP alone but was enhanced 5-10 fold by priming (Figure 6.1). A small (but statistically significant) increase in $\text{Ins}(1,4,5)\text{P}_3$ accumulation in response to 1 nM fMLP was seen in PAF- and $\text{TNF}\alpha$ -primed cells at 10 s (Figures 6.3 and 6.4); however, neither agent significantly affected the time-course (0-10 min) of $\text{Ins}(1,4,5)\text{P}_3$ accumulation induced by either 1 or 100 nM fMLP (Figures 6.5-6.8 and Table 6.1). These data render an important role for $\text{Ins}(1,4,5)\text{P}_3$ in neutrophil priming unlikely. All these studies, however,

relate to neutrophil populations; when analysed on an individual basis, neutrophils demonstrate heterogeneity on multiple levels (Gallin, 1984) including Ca^{2+} signalling (Yee and Christou, 1993, Elsner et al., 1992) and respiratory burst activity in unprimed (Fritzsche and de Weck, 1988) and primed neutrophils (Elbim et al., 1994). It is therefore possible (though unlikely) that enhanced responses in a subpopulation of cells were masked by unaffected cells.

In company with $\text{Ins}(1,4,5)\text{P}_3$ signalling, the recently elucidated $\text{PtdIns}(3,4,5)\text{P}_3$ signal transduction pathway is critical to the generation of the neutrophil respiratory burst (Dewald et al., 1988). Additionally, activation of the small GTP-binding protein p21^{rac} , which is an essential component of the NADPH oxidase, has been shown to lie downstream from $\text{PtdIns}(3,4,5)\text{P}_3$ in PDGF-stimulated membrane ruffling in an endothelial cell line (Hawkins et al., 1995). Since $\text{TNF}\alpha$ does not elicit $\text{PtdIns}(3,4,5)\text{P}_3$ accumulation in human neutrophils (L. Stephens, personal communication), we elected to study the effect of preincubation with $\text{TNF}\alpha$ on the fMLP-stimulated $\text{PtdIns}(3,4,5)\text{P}_3$ signal. It was clearly demonstrated that $\text{TNF}\alpha$ augmented the accumulation of $\text{PtdIns}(3,4,5)\text{P}_3$ generated in response to fMLP stimulation. This effect was small but significant 10 s following the addition of fMLP (an enhancement of $144\pm 11\%$ in primed versus unprimed cells, Figure 7.6.A) but by 1 min after fMLP stimulation, a time corresponding to peak O_2^- generation (Christiansen, 1988 and L. Kitchen, personal communication) this enhancement was $620\pm 74\%$ (Figure 7.6.B). The enhancement of O_2^- generation from the pooled experiments was $393\pm 15\%$, suggesting a potentially important role for the prolonged and augmented accumulation of $\text{PtdIns}(3,4,5)\text{P}_3$ in the $\text{TNF}\alpha$ -primed, fMLP-stimulated respiratory burst. In the same experiments, changes in the detection of $\text{PtdIns}(3,4,5)\text{P}_3$ were closely paralleled by changes in its immediate

metabolite, PtdIns(3,4)P₂, suggesting that the augmented PtdIns(3,4,5)P₃ accumulation is not secondary to decreased metabolism.

In view of the wide spectrum of priming agents, it is possible that their influence on the final common pathway of NADPH oxidase activation may occur via differing routes. For example, it has been speculated that PtdIns(3,4,5)P₃ may promote dissociation of p21^{rac} from its inhibitor, rhoGDI, thereby enhancing p21^{rac} translocation to the neutrophil membrane; other biologically active lipids, including arachidonic acid, also disrupt the complexation of rac with GDI (Chuang et al., 1993), thus activators of PLA₂ could also potentially regulate NADPH oxidase activity via this route. Phosphorylation of the oxidase component p47^{phox} is an important step in respiratory burst activation which is potentially regulated by a number of tyrosine kinases, including MAPK; the extensive literature on this subject has been summarised by Hallett and Lloyds (1995), and has not been further addressed in this thesis.

In conclusion, the work presented in this thesis has demonstrated that priming agents regulate neutrophil membrane adhesion molecule and G_{iα2} expression, but do not influence Ins(1,4,5)P₃ signalling; the priming agent TNFα markedly enhanced fMLP-stimulated PtdIns(3,4,5)P₃ accumulation, an effect which may be relevant to the membrane translocation of p21^{rac}, an obligatory component of the NADPH oxidase.

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